Structure and Expression of the Murine Periaxin Gene

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DECLARATION

I declare that this thesis was composed by myself and that the work described within it is my own, except where stated.

Lee M. Dytrych
September 1997

This thesis is dedicated to Margaret Dytrych.
I know you'd be proud, mam.

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Immense thanks must go to Peter Brophy, a supervisor who was there when I needed him, with support and great suggestions. I am especially grateful to Stewart Gillespie who taught me all there is to know about pipettes and Eppendorf tubes (not to mention the horrors of junior league football and the delicacy that is a Perfect Pizza). Thanks must also go to the many friends that have passed through the Brophy' lab over the last four years and who have made this work as enjoyable as it was.

I am very grateful to the BBSRC for funding this work and to the Vet school for their financial support during my conference trip to San Francisco.

I could never have achieved so much without the love and support of my family.

Finally, words can't even begin to express just how important you've been, Tan.

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To all the people who have been there through the good and bad times I thank you from the bottom of my heart.

FIGURES

TABLES

AMINO ACID ABBREVIATIONS

Alanine	**Ala**	**A**
Arginine	**Arg**	**R**
Asparagine	**Asn**	**B**
Aspartic acid	**Asp**	**D**
Cysteine	**Cys**	**C**
Glutamine	**Gln**	**Q**
Glutamic acid	**Glu**	**E**
Glycine	**Gly**	**G**
Histidine	**His**	**H**
Isoleucine	**Ile**	**I**
Leucine	**Leu**	**L**
Lysine	**Lys**	**K**
Methionine	**Met**	**M**
Phenylalanine	**Phe**	**F**
Proline	**Pro**	**P**
Serine	**Ser**	**S**
Threonine	**Thr**	**T**
Tryptophan	**Trp**	**W**
Tyrosine	**Tyr**	**Y**
Valine	**Val**	**V**

ABBREVIATIONS

A	Adenine
bp	Base pair
cDNA	Copy DNA
C	Cytosine
CGT	Ceramide galactosyltransferase
CMT	Charcot-Marie-Tooth neuropathy
CNPase	2',3'-cyclic nucleotide-3'-phosphodiesterase
CNS	Central nervous system
Cx32	Connexin32
DNA	Deoxyribonucleic acid
DSS	Dejerine-Sottas syndrome
DTT	Dithiothreitol
EtBr	Ethidium bromide
EDTA	Ethylene diamine tetra acetic acid
ER	Endoplasmic reticulum
FABP	Fatty acid binding protein
G	Guanine
GalC	Galactocerebroside
Golli	Gene expressed in the oligodendrocyte lineage
HNPP	Hereditary neuropathy with liabilty to pressure palsies
IgG	Immunoglobulin G
IPL	Intraperiod line
kb	Kilobase
kD	KiloDalton
KLH	Keyhole limpet haemocyanin
L-Peri	Large (147 kDa) periaxin isoform
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MDL	Major dense line
Mr	Molecular weight
mRNA	messenger RNA
MS	Multiple sclerosis
nt	nucleotide
NCAM	Neural cell adhesion molecule

P2	Myelin protein-2
PBS	Phosphate buffered saline
pfu	Plaque forming units
PLP	Proteolipid protein
PMP22	Peripheral myelin protein-22
PNS	Peripheral nervous system
P0	Peripheral myelin protein zero
Prx	Periaxin gene
RACE	Rapid amplification of cDNA ends
RNase	Ribonuclease
RNA	Ribonucleic acid
rpm	Revolutions per minute
SCIP	Suppressed cAMP Inducible POU
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S-Peri	Small (16 kDa) periaxin isoform
T	Thymine
TCA	Trichloroacetic acid
Tr	Trembler
UTR	Untranslated region

ABSTRACT

Periaxin was first identified as a 147 kDa protein which was subsequently found to be a relatively abundant component of myelinating Schwann cells. Periaxin displays a pattern of expression that is highly regulated both spatially and temporally in the developing peripheral nervous system of the rat. After the protein's initial localization to the periaxonal membranes of Schwann cells as they begin axonal ensheathment (hence the name periaxin), it becomes predominantly abaxonal as the myelin sheaths mature. This shift in the localization of the protein in the Schwann cell after completion of the spiralization phase of myelination suggests that periaxin may participate in the membrane-protein interactions that are required to stabilize the mature sheath.

Although earlier studies had been focused on rat periaxin, in this thesis the structure of the mouse *periaxin* gene (*Prx*) has been determined. This was in order to facilitate further studies on the molecular genetics of periaxin expression and function. Analysis of the structure and expression of the mouse *periaxin* gene has revealed that the gene encodes two polypeptides and the mechanism by which these two proteins are encoded has been established. Furthermore, the localization of these two proteins has been shown to differ in myelinating Schwann cells.

A mouse genomic library constructed in the bacteriophage replacement vector Lambda Dash II was screened by plaque in situ hybridization using a full length rat periaxin cDNA as probe. Clones covering the entire length of the gene were isolated and fully characterized. The murine *periaxin* gene spans 20.6 kb, includes seven exons and encodes two mRNAs of 4.6 and 5.2 kb. The largest of these exons is 4 kb in length and encodes 90% of the protein coding sequence and all of the 3' untranslated region.

Sequence analysis of the gene's core promoter identified several interesting features. A TATA-box motif is absent; hence the murine *periaxin* gene promoter

belongs to the TATA-less family. Direct comparison with the promoters of myelin genes expressed in Schwann cells detected several common motifs, the most important of which is known to bind the transcription factor SCIP/Oct-6. This factor has been shown to have a vital role in the regulation of myelination by Schwann cells. Such features underline the potential importance of periaxin in the myelination process.

The 4.6 kb mRNA includes all seven exons and encodes the previously described protein of 147 kDa (now termed L-periaxin). The 5.2 kb mRNA, in addition to the seven exons, also includes an intron comprising 600 bases located between exon 6 and 7. The inclusion of this intron introduces both novel amino acid sequence and a new stop codon and results in the translation of a truncated form of the periaxin protein with a unique C-terminus which was previously undetected. During post-natal development of the sciatic nerve, the level of expression of this protein (named S-periaxin) was shown to parallel that of L-periaxin. In contrast, the localization of the S-periaxin, determined by indirect immunofluorescence, was neither periaxonal nor abaxonal, but restricted to the Schwann cell cytoplasm.

The differential localization of L-periaxin and S-periaxin indicates that they serve distinct functions in the myelinating Schwann cell.

Introduction

1.1 INTRODUCTION

The past two decades has seen an explosion of new information about the organization, structure, and regulation of genes in eukaryotic organisms. The reason for this enormous increase in our knowledge has been the concurrent development of recombinant DNA techniques and methods for determining nucleotide sequences. Despite these great advances however, our understanding of the exact genetic processes involved in the growth and development of highly differentiated multicellular organisms remains fragmentary at best and is most likely still naive.

Given that each cell contains the same complement of genes and originates from a single fertilized egg, a major challenge is to understand how different types of cells express widely different proteins. In addition, these differential patterns of protein synthesis occur at different developmental stages of the same cell. Therefore, the mechanisms of gene regulation which exist in eukaryotes must be exquisitely intricate and complicated. So much so in fact, that these mechanisms are not understood for even one mammalian gene to the extent that they are understood for many prokaryotic operons. Clearly, the task for researchers is a difficult one. Sequencing the entire genome of an organism will rapidly provide the knowledge of the individual constituents, but showing how the expression of each and every one is integrated and coordinated with respect to the whole animal is a feat which may take many years.

The most sensible way forward perhaps, is to consider a multicellular organism not as a whole but more as a sum of its parts. In other words, determine the developmental processes of the individual cell types. It should then be possible to discern the underlying mechanisms, thus providing a basis for a complete understanding of the entire system. The Schwann cell, the myelin-forming cell of the peripheral nervous system of higher vertebrates, is an ideal cell type to study cellular differentiation given its distinctive developmental phenotype and the

associated highly coordinated, highly selective pattern of gene expression. Characterization of the programme of gene expression involved in the myelination process will also be of tremendous value to those seeking to understand, at the molecular level, the large number of neurological diseases caused by defects in the myelin sheath. Ultimately, it is hoped that such information will lead to new therapies. The following is therefore a review of our current knowledge of Schwann cell development, differentiation and myelination and the molecular basis thereof.

1.2 THE SCHWANN CELL AND MYELINATION

1.2.1 An Introduction to Myelin

The rapidly conducting nerve fibres of higher vertebrate animals possess a segmental myelin sheath which is a lipid-rich, multilamellar organelle (Morell, 1984). This substance serves principally as an electrical insulator, greatly increasing the velocity of impulse propagation by facilitating saltatory conduction (Ranvier, 1878; Stampfli, 1981; Ritchie, 1984, 1987; Waxman and Ritchie, 1985). To this purpose, myelin is found in both the central nervous system (CNS) and in the peripheral nervous system (PNS). In the CNS, myelin is elaborated by highly specialized, non-neuronal cells known as oligodendrocytes (Bunge et al, 1962). Importantly, the myelin-forming cells of the PNS comprise a completely different lineage and are known as Schwann cells (Geren, 1954). Despite this fact, both cell types form myelin by repeatedly wrapping a flattened extension of their plasma membrane in a spiral around the nerve axon. The subsequent extrusion of cytoplasm from within the spiral, leads to the apposition of both adjacent cytoplasmic membrane surfaces and adjacent extracellular membrane surfaces, forming mature compact myelin. At the macroscopic level therefore, CNS and PNS myelin are very similar (Kirschner et al, 1984; Readhead et al, 1987). However, this is not the case at the molecular level,

where striking compositional differences between the two myelins are observed. These compositional differences occur primarily within the complement of myelin (and myelin-associated) proteins utilized by each cell type, and whose functional properties collectively bring about the formation and maintenance of the respective multilamellar structures. To a certain extent therefore, Schwann cell myelin and oligodendrocyte myelin can be considered an example of convergent evolution, much like the echolocation of bats and dolphins - the same function, brought about in a similar way, but developed independently. Thus, PNS and CNS myelin, and the cells responsible for their elaboration, must be treated as distinct but related entities.

1.2.2 From Neural Crest to Schwann Cell

Like the peripheral neurons they are destined to myelinate, Schwann cells have their origins in the neural crest (Le Douarin, 1982, 1986; Le Douarin et al, 1991; Fraser and Bronner-Fraser, 1991; Frank and Sanes, 1991; Anderson, 1993). This transient and relatively small, pluripotent population of cells located dorsal to the neural tube (the origin of oligodendrocytes [Pringle and Richardson, 1993]), migrate widely throughout the embryo during development (Weston, 1963; Rickmann et al, 1985; Bronner-Fraser, 1986; Loring and Erickson, 1987), and give rise to a number of other distinct cell types including melanocytes, smooth muscle cells, endocrine cells, and osteoblasts. Crest cells destined to follow the Schwann cell lineage migrate into the PNS at a very early stage of development (Le Douarin et al, 1991; Stemple and Anderson, 1992) and become closely associated with embryonic peripheral nerves (Dahm and Landmesser, 1988). Concomitant with this process, these cells undergo extensive differentiation (Jessen et al, 1994; Dong et al, 1995; Gavrilovic et al, 1995), forming Schwann cell "precursors", which represent a distinct intermediate in the generation of Schwann cells from neural crest cells (Jessen et al, 1994) (Figure 1).

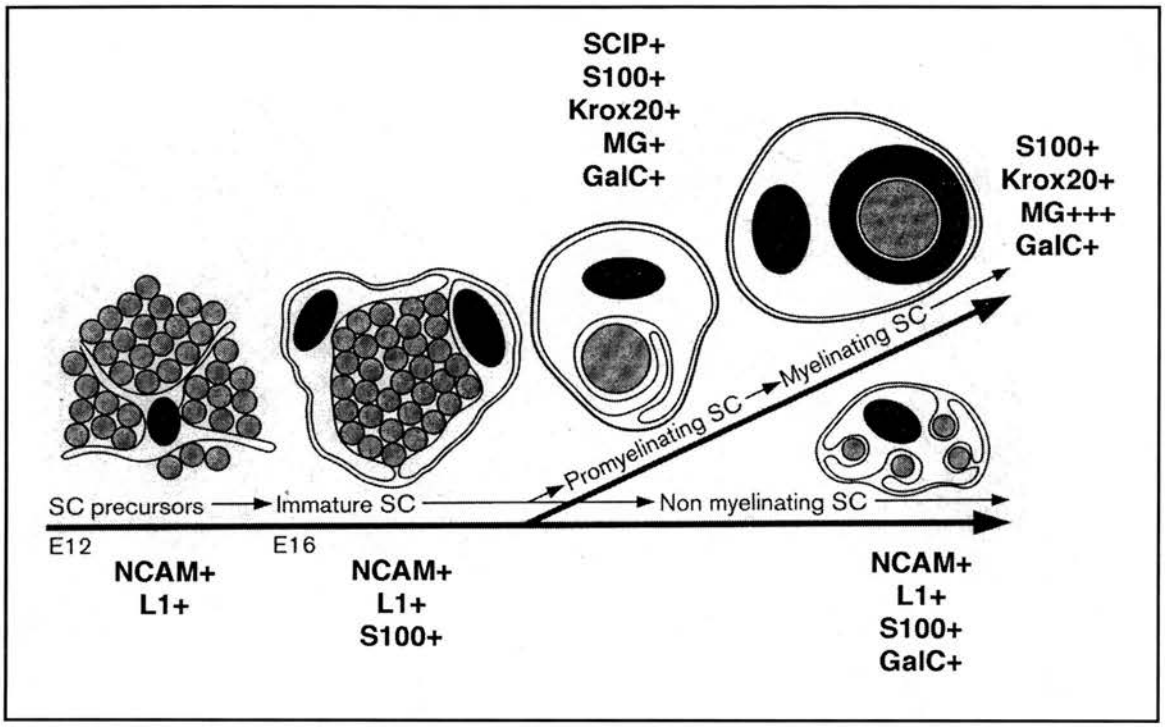


Figure 1. Stages in Schwann cell (SC) differentiation. This diagram summarises the profile of markers used to define distinct developmental stages in both myelinating and non myelinating Schwann cell lineages. Markers are as follows: NCAM, neural cell adhesion molecule; L1, an immunoglobulin-related adhesion protein; S100, a Schwann cell calcium binding protein; Krox20 and SCIP, transcription factors; GalC, galactocerebroside; MG, Myelin structural proteins. After Scherer (1997).

Interestingly, Schwann cell precursors maintain certain crest cell features including a flattened morphology and high motility. The importance of such characteristics is demonstrated by a rapid phase in which these cells begin to invade large nerve bundles (Peters and Muir, 1959; Gamble and Breathnach, 1965; Gamble, 1966; Ziskind-Conhaim, 1988), extending finger-like processes which progressively subdivide, segregate and envelop the individual axons (Ashbury, 1975; Raine, 1984). In the rat sciatic nerve, this takes place during embryonic day 14 to 15 (E14-E15) (Jessen et al, 1994). Each Schwann cell precursor eventually forms processes around many axons which, at this stage of development, are relatively thin (0.2-0.5 μm). As the cells continue to proliferate, the number of axons associated with each cell decreases. By E16/E17 each cell is associated with relatively few axons which lie within separate furrows, indenting the outer cell membrane (Webster, 1971). Phenotypically, the cells at this stage are radically different from the precursors (Jessen et al, 1994), particularly with respect to the antigenic markers they express which are more typical of mature, fully differentiated Schwann cells e.g. S100 (a calcium binding protein) and the O4 (sulphatide) antigen (Sommer and Schachner, 1981; Jessen et al, 1990; Mirsky et al, 1990). These cells are thus defined as embryonic Schwann cells (Figure 1).

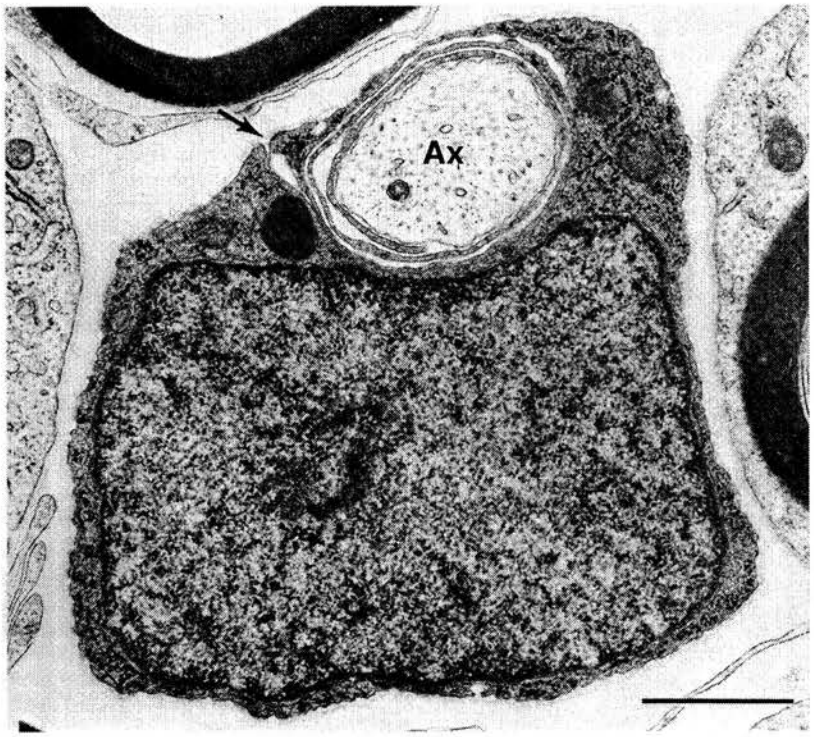
As development progresses, embryonic Schwann cells continue to divide, transferring axons to their progeny after each division until a 1:1 Schwann cell:axon relationship is reached. Subsequently, these cells exit the cell cycle and synthesize a basal lamina (Bunge et al, 1982; Bunge et al, 1986). Progression from this promyelinating phenotype to mature myelin-forming Schwann cells occurs in the first week of post-natal life in the rodent sciatic nerve, and within the third foetal trimester in humans, and is associated with the high level synthesis of myelin-associated proteins and membrane components (Webster, 1971; Wood and Engel, 1976; Lemke and Axel, 1985; Lemke and Chao, 1988; Willison et al, 1987; Trapp et

al, 1988) and the downregulation of embryonic Schwann cell markers (Lemke and Chao, 1988; Taniuchi et al, 1988; Jessen et al, 1990).

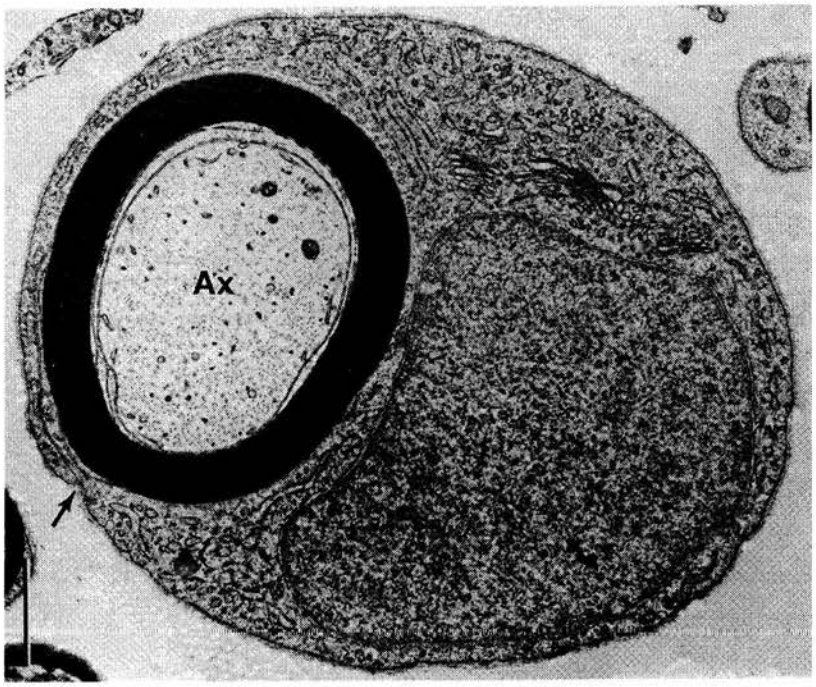
The commencement of myelin formation is first visualized at the membrane bound lip on one side of the neck of the Schwann cell mesaxon (the extracellular space connecting the axonal furrow to the cell surface), which flattens and then tucks under the opposite side (Webster, 1971) (Figure 2A). This cytoplasmic process then progresses in a spiral around the surface of the axon, displacing the previous turns (Bunge et al, 1989; Webster and Favilla, 1984). Once 2-6 turns are elaborated membrane compaction begins (Peters and Muir, 1959; Webster, 1971; Webster and Favilla, 1984), giving rise eventually to a mature myelin segment (Figure 2B).

In all developing nerves, a small population of embryonic Schwann cells fail to form myelin, and instead develop a non myelinating phenotype. These cells retain many characteristics of the embryonic Schwann cell, including association with multiple thin axons (Ochoa, 1976; Webster and Favilla, 1984; Pannese et al, 1988), the expression of shared antigenic markers and importantly, they do not upregulate myelin proteins (Brockes et al, 1979; Jessen and Mirsky, 1991; Mirsky and Jessen, 1996). However, they do acquire the cell-surface marker galactocerebroside (GalC) (Jessen and Mirsky, 1985; Jessen et al, 1987). The choice between a myelin-forming phenotype or this non myelin-forming phenotype has been shown to be determined by the axons with which the cells find themselves in contact (Aguayo et al, 1976; Weinberg and Spencer, 1976; Lemke and Chao, 1988; Voyvodic, 1989). Indeed, initiation of a myelinating fate is only observed in embryonic Schwann cells that associate with axons that grow larger than 1 μm (Peters and Vaughn, 1970; Martin and Webster, 1973; Webster et al, 1973); the multiple axons associated with non myelin-forming Schwann cells are significantly thinner. This is further supported by the finding that non myelinating Schwann cells are able to differentiate into a myelin-forming state when transplanted into a nerve

A.



B.



that normally contains many myelinating Schwann cells (Langley and Anderson, 1903; Simpson and Young, 1945; Aguayo et al, 1976; Weinberg and Spencer, 1976). It is clear therefore, that all embryonic Schwann cells have the potential to form myelin, but they will do so only upon induction by signals from appropriate peripheral axons.

Although the molecular nature of these signalling molecules remains unknown, they are likely to act in part by activating the adenyl cyclase cytoplasmic pathway within the Schwann cell, since the presence of agents that elevate intracellular cAMP levels (e.g. forskolin, dibutyryl cAMP and cholera toxin) in Schwann cell cultures lacking neuronal support, mimics many of the effects of axonal contact (Sobue and Pleasure, 1984; Lemke and Chao, 1988; Schuman et al, 1988; Trapp et al, 1988; Monuki et al, 1989; Morgan et al, 1991; Jessen et al, 1991), in particular the induction of myelin-specific genes. A long gap (> 12 hours) between intracellular cAMP elevation and gene activation is observed in such cells, suggesting that this induction occurs indirectly, most probably via a cascade of intermediate, cAMP-induced regulatory molecules (Lemke and Chao, 1988; Morgan et al, 1994).

From the cell-specific induction of myelin formation and the synchronized nature of the myelinating events it is evident that myelination is subject to an extremely high level of control. As shown, the role of axons in this regulation cannot be understated, with axonal contact an essential prequisite for terminal differentiation of Schwann cells and the maintenance of this state (Wood and Bunge, 1975; Salzer and Bunge, 1980; De Vries et al, 1982; Pleasure et al, 1985; Jessen and Mirsky, 1991). Schwann cell-axon contact is also thought to be vital in the progression from precursor to embryonic Schwann cell, communicating signals which dictate survival, proliferation and the timing of maturation (Zorick and Lemke, 1996; Topilko et al, 1996; Mirsky and Jessen, 1996). Research aimed at deciphering the precise nature of these molecular signals is growing rapidly in importance. Given that cell-cell contact and communication is a vital component of

the coordinated development of most body systems not just the PNS this is hardly surprising.

1.3 THE STRUCTURE OF PNS MYELIN

1.3.1 The Segmental Nature of the Myelin Sheath

Myelinated PNS fibres possess a highly distinctive morphology, with myelin clearly discernible as a segmental sheath around the axon by light microscopy even at low power. Each individual myelin segment (known as an internode) is generated by a separate Schwann cell (Geren, 1954; Webster, 1971). The entire length of the axon is therefore myelinated by a chain of Schwann cells lying parallel to the fibre. Importantly, this discontinuity in the way myelin is laid down i.e. as consecutive internodes, is the key to rapid saltatory nerve impulse propagation (Stampfli, 1981; Ritchie, 1984; Waxman and Ritchie, 1985; Ritchie, 1987). Adjacent internodes do not abut one another directly but instead are separated by a small region of axonal membrane which remains bare (Figure 3). These are the nodes of Ranvier (Ranvier, 1871), which are the foci of all electrical activity, with practically all of the axonal Na^+ and K^+ ion channels being concentrated in these regions. When an action potential is triggered at a node, the local current generated cannot flow through the adjacent membrane due to the high electrical resistance of the myelin. Instead, the current can only leave at the next node. As this node depolarizes, enough current is generated to excite the next more distant node. Because the excitation of the membrane therefore jumps from node to node, the conduction velocity of impulses is significantly faster than that of unmyelinated nerve fibres; myelinated axons conduct impulses about 10x faster than unmyelinated axons of a comparable calibre (for review see Ritchie, 1984).

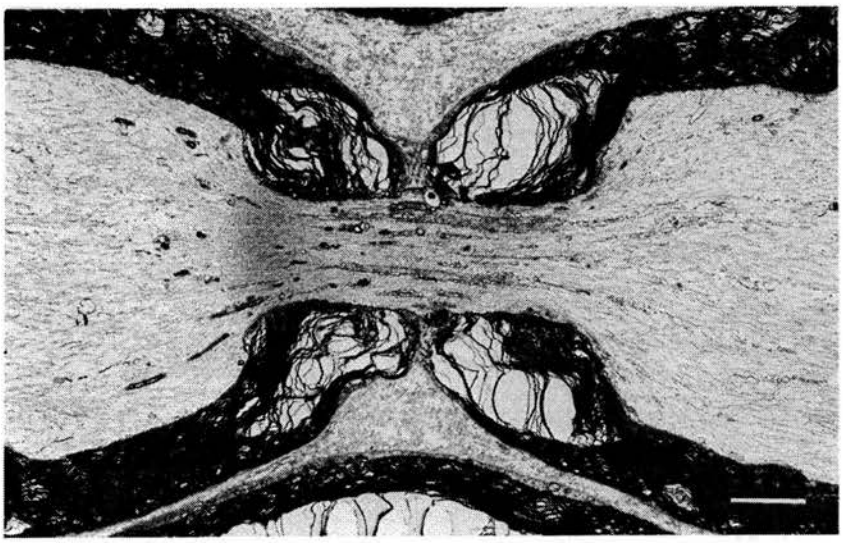


Figure 3. PNS node of Ranvier of a large diameter axon from a rabbit anterior spinal nerve root. Note the lateral termination of the loops of Schwann cell cytoplasm and the condensation of axoplasmic organelles within and to one side of the node. From Raine (1984).

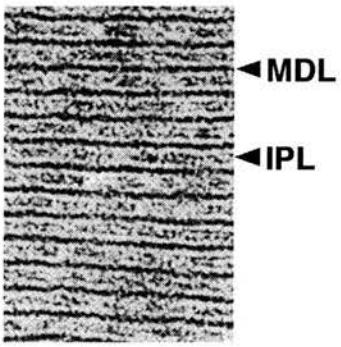


Figure 4. Cross section through mature PNS myelin showing its multilamellar structure. During compaction, the major dense line (MDL) is formed by the apposition of adjacent cytoplasmic membrane surfaces, whereas the intraperiod line (IPL) is formed by the apposition of adjacent intracellular membrane surfaces. From Raine (1984).

1.3.2 Compact Myelin

Schwann cells elaborate myelin by repeatedly wrapping a flattened extension of their plasma membrane in a spiral around the nerve axon. The subsequent extrusion of cytoplasm from within this spiral leads to the apposition of the membrane bilayers, forming mature compact myelin. These apposed membrane surfaces are highly visible on electron micrographs of myelin in cross section where they appear as regularly alternating light and dark bands (Napolitano and Scallen, 1969; Peterson and Pease, 1972) (Figure 4): the intraperiod line is derived from the fusion of the extracellular membrane surfaces and the major dense line is derived from the fusion of the intracellular membrane surfaces. Like counting the growth rings on a tree trunk, it is possible to calculate the number of times that the Schwann cell has encircled the axon by counting these lines. Interestingly, there is a direct correlation between the thickness of the axon and the number of membrane turns (and hence the thickness of the sheath), with large diameter axons possessing thicker myelin than thinner ones (Friede and Samorajski, 1967; Matthews, 1968; Friede, 1972; Friede and Bischhausen, 1982). Once again this provides good evidence for the existence of axonal regulatory influences over Schwann cells and myelination.

1.3.3 Uncompacted Myelin

Although myelin is essentially a compact structure, Schwann cell cytoplasm-filled regions do persist, most notably within the periaxonal and adaxonal spaces, within the paranodal loops (Figure 5) and within Schmidt-Lanterman incisures (Figure 6). These morphological features define regions of imperfect myelin compaction where the adjacent cytoplasmic membranes have failed to abut. The necessity for retaining these cytoplasm-filled structures remains unclear, but given that they occur in the regions of direct cell-cell contact an involvement in intercellular communication has been suggested.

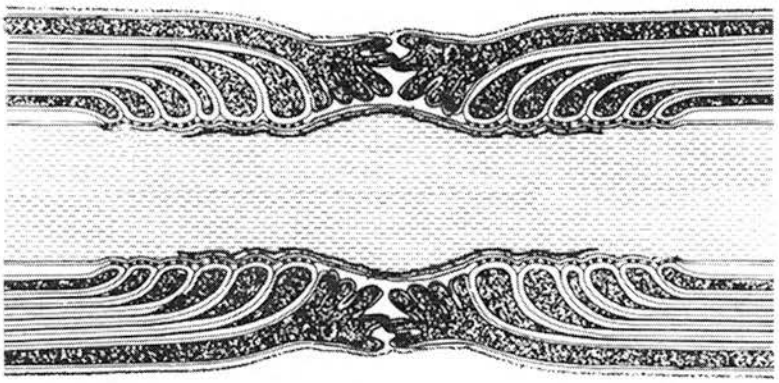


Figure 5. Diagram of the PNS node showing the regimented organisation of the paranodal loops. These lateral specializations of the myelin sheath form tight (glial-glial) junctions with neighbouring loops. Because of their proximity to the excitable axolemma of the node of Ranvier, the paranodal loops may be involved in the ion fluxes that occur during nerve impulse propagation (or axon-glial communication). From Raine (1984).

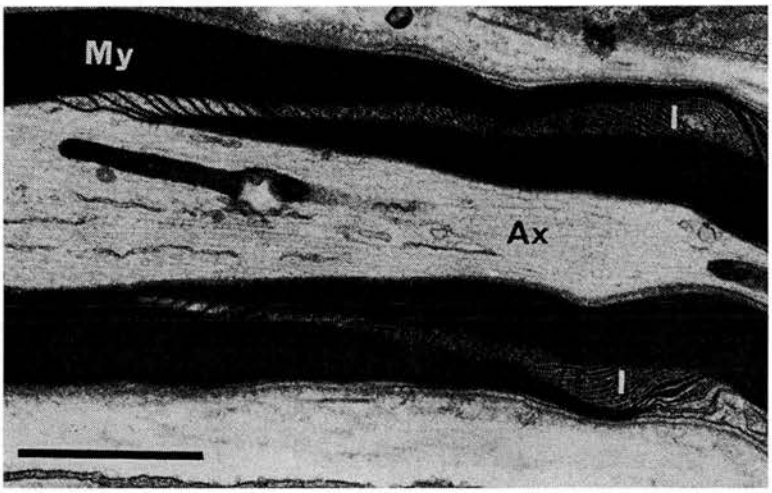


Figure 6. Electron micrograph of longitudinal section through a myelinated nerve fibre from an adult human sural nerve showing a Schmidt-Lanterman incisure (I) traversing the myelin sheath. These cytoplasm filled channels remain due to imperfect compaction of the major dense line. Often they provide a direct connection between the Schwann cell body and the periaxonal region. Thus, these channels are pefectly suited to function as a transport network throughout the compact myelin sheath. From Dyck et al (1993).

1.4 THE BIOCHEMISTRY AND MOLECULAR BIOLOGY OF PNS MYELIN

Myelin is an extension of a eukaryotic plasma membrane. Therefore, like the plasma membrane (and all other cellular membranes), myelin is composed of a heterogenous group of lipids and proteins whose interactions and associations - both within the membrane and with the underlying cytoskeleton - determine the macroscopic structure of the organelle. Given that myelin is structurally highly distinct, it is reasonable to presume that the lipids and proteins of which it is constructed must also be highly distinct. Indeed, it has been clear for many years that the chemical composition of the myelin membrane is remarkably different to the cellular membrane from which it originates (see Smith, 1983; Morell, 1984 for reviews). This composition is undoubtedly fundamentally related to the process of myelinogenesis and to the unique biochemical and biophysical properties of myelin which are essential for its role as an insulator of nerve fibres and mediator of saltatory nerve impulse conduction.

1.4.1 PNS Myelin Lipids

The dominant feature of myelin composition relative to other cell-surface or intracellular membranes is the high ratio of lipid to protein. Typically, cellular membranes contain approximately equal amounts of proteins and lipids (Finean et al, 1984), but in myelin, protein may be as little as 20% of the total dry mass (Smith, 1983). In purely functional terms, this is unsurprising given that the major role of myelin is to provide high-resistance, low capacitance insulation around the nerve (Ritchie, 1984). Since a high concentration of lipid effectively restricts the passive flow of water and conducting ions (Na^+, K^+), the lipid content of myelin contributes to these electrical properties essential to myelin's function as an insulator. However, although the lipids of myelin supply the physical properties

necessary for this insulating barrier, their known heterogeneity is a clear indication of a more dynamic role, particularly during assembly and maintenance of the myelin sheath (for reviews see Morell, 1984; Martenson, 1992).

The lipid composition of myelin has been extensively studied in a great many species (reviewed by Norton and Cammer, 1984). As for cell membranes generally, the major lipid constituents of myelin membrane are cholesterol, phospholipids, and glycolipids (Table 1).

(a) Cholesterol

On the basis of weight percent, cholesterol is the most abundant single lipid molecule, comprising about 25%. This figure is similar in both mammalian and non-mammalian species (Norton and Cammer, 1984) and is comparable to the values given for most eukaryotic plasma membranes (Finean, 1984).

Functionally, cholesterol is believed to be an important determinant of membrane fluidity via interactions with the hydrocarbon chains of the bilayer (Benga and Holmes, 1984). In addition, cholesterol decreases the permeability of lipid bilayers to small water soluble molecules and is thought to enhance both the flexibility and the mechanical stability of the bilayer.

(b) Phospholipids

Second to cholesterol, the next most abundant lipid components of the myelin bilayer are phospholipids (Table 1). These comprise four major families of lipid species: the phosphatidylethanolamines, the phosphatidylcholines, the phosphatidylserines and the sphingomyelins. Collectively these lipids constitute more than half the mass of lipids, outnumbering cholesterol molecules nearly 2:1 (although individually no species predominates outright). Across animal species, the phospholipid composition of myelin is much the same, with a few exceptions. For example, rat myelin has much less sphingomyelin than bovine or human myelin

TABLE 1

Composition of PNS myelin from several species (Norton and Cammer, 1984)

Component	Rat (Smith and Curtis, 1979)	Human (Spritz et al, 1973)	Bovine (O'Brien et al, 1967)
Total Protein[b]	-a	28.7	24.1
Total Lipid	-a	71.3	75.9
Cholesterol[c]	27.2	23.0	24.7
Total Glycolipid[c]	21.5	22.1	16.6
Galactocerebroside[c]	15.8	-a	14.1
Sulfatide[c]	5.7	-a	2.5
Total Phospholipid[c]	50.6	54.9	58.2
PT Ethanolamine[c,d,e]	19.2	19.2	16.3
PT Choline[c,d]	9.6	8.2	12.2
PT Serine[c,d]	11.1		7.6
PT Inositol[c,d]	2.5	9.3f	-a
Sphingomyelin[c,d]	8.6	18.7	15.7

[a](-) Not analysed

[b] Total protein and total lipid expressed as percentage of dry weight

[c] Figures are expressed as percentage of total lipid

[d] (PT) Phosphatidyl

[e] Mostly ethanolamine plasmalogen (Horrocks, 1972)

[f] PT serine and PT inositol were analysed together.

(Table 1) and is more comparable to that of plasma membranes generally. Other phospholipid families such as the phophatidylinositols are also present in PNS myelin but in relatively minor quantities.

The presence of such a wide variety of phospholipids, whose head groups differ in size, shape, and charge suggests that these molecules are involved in important interactions. Further evidence for an active role in myelin structure is provided by the intriguing finding that the different families of phospholipids are known to be asymmetrically distributed within the bilayer: lipid molecules that have choline in their head group (sphingomyelin and phosphatidylcholine) are predominantly found in the outer, extracellular half of the bilayer, while phospholipid molecules that contain a terminal primary amino group (phosphatidylethanomlamine, phosphatidylserine and phosphatidylinositol) predominantly reside in the inner half (Zachowski and Devaux, 1989; Kirschner and Ganser, 1982). Asymmetry of these molecules therefore generates asymmetry in the biochemical and biophysical nature of the lipid bilayer. Although the function of this asymmetry is largely unknown it has been postulated to reflect the requirements of proteins associated with the membrane, particularly the cytoskeleton (for review see Brophy, 1992).

(c) Glycolipids

The remaining lipid component of myelin are the glycolipids (Table 1). Galactocerebroside (GalC) and its sulphate derivatives (sulphatides) represent upwards of 20% of the dry weight of PNS myelin lipids from most species (Radin, 1983; Kishimoto, 1983; Norton and Cammer, 1984). In other plasma membranes this figure is seldom greater than 5% (Finean, 1984). Indeed, although no lipids are 'myelin-specific' this enrichment of GalC and sulphatide is one of few distinguishing features of myelin with regards to lipid composition. Like the phospholipids, the glycolipids are asymmetrically distributed within the myelin membrane, and are

found only in the outer half of the bilayer with their sugar moieties exposed at the cell surface. PNS myelin also contains minor quantities of complex glycolipids known as gangliosides (Fong et al, 1976), which are distinguished by a sugar chain and sialic acid. Because of the growing knowledge that glycolipids (particularly those containing sialic acid residues) are important in a wide variety of cell surface phenomena including cell-cell and membrane-membrane interactions, it has been suggested that their presence may be functionally significant during myelinogenesis (Wiegandt, 1982). Unfortunately, for GalC and its derivatives at least, this may not be true: a number of groups have recently developed null mouse mutants lacking the enzyme UDP-galactose: ceramide galactosyltransferase (CGT) which is responsible for the synthesis of GalC. Although these animals form myelin with an apparently normal ultrastructure they display a relatively severe clinical phenotype with progressive demyelination (Coetzee et al, 1996; Bosio et al, 1996). This indicates that GalC and/or sulphatide may be involved in myelin maintenance and function, but not ensheathment.

Given that many of the compositional characteristics of myelin in regards to its major lipid constituents are similar to those of eukaryotic plasma membranes in general it is difficult to assign any lipid species a specific role in myelinogenesis. Furthermore, other naturally occurring membrane assemblies that exhibit orderly and close stacking like myelin, such as rod outer segment disk membranes (Chabre, 1975; Daemon, 1973) and the grana thylakoid membranes of the chloroplast (Li, 1978), display distinctly different compositional characteristics. Neither assembly has appreciable amounts of cholesterol (Daemon, 1973; Li, 1978). Moreover, while the rod disk membranes contain very little glycolipid, the grana membrane lipids are nearly two-thirds glycolipid by weight. From these findings, it would appear that the multilamellar stacking in myelin and these other systems does not correlate with a particular lipid composition. The specific membrane interactions in myelin are therefore likely to be brought about and modulated by the particular protein

components, with the lipid heterogeneity simply reflecting the requirements of these components.

1.4.2 PNS Myelin Proteins

As discussed, the protein content of myelin membrane is consistently lower than that of other cell-surface or intracellular membranes (Smith, 1983). However, this does not mean that the protein constituents are relatively unimportant, far from it. While the lipids are responsible for the function of myelin ie insulating nerve axons, it is the proteins which determine the structure via their specific molecular interactions (for reviews see Morell, 1984; Sutcliffe, 1987; Mikoshiba et al, 1991; Lemke, 1988; 1993). A great deal of effort and time has therefore been spent characterizing the protein constituents of myelin membrane with the belief that these molecules hold the key to the understanding of the myelination process.

Due to the extreme susceptibility of purified PNS myelin to proteolysis only the most abundant and/or resistant proteins have been studied in any great depth (Norton and Cammer, 1984). These major proteins are glial cell-specific (in particular myelinating glia-specific) and are primarily structural in nature. Furthermore, these proteins tend to be highly conserved among the higher vertebrate species, suggesting essential roles in the formation and maintenance of myelin.

(a) Peripheral Myelin Protein Zero (P0)

P0 is the single most abundant protein of PNS myelin, accounting for roughly 40-50% of the total protein content (Greenfield et al, 1973; Ishaque et al, 1980). Although it is expressed at such high levels in the PNS, P0 protein or its mRNA has not been detected in the CNS. P0 protein is further restricted within the PNS itself, being expressed wholly by myelinating Schwann cells (Lees and Brostoff, 1984; Politis et al, 1982). In particular, it is predominantly localized to the compact

myelin formed by these cells, where it is believed to be responsible for the formation and maintenance of both the intraperiod and the major dense lines.

First identified by lectin binding studies carried out on PNS myelin (Everly et al, 1973; Wood and Dawson, 1973; Brostoff et al, 1975; Kitamura et al, 1976), P0 is a glycoprotein. The full sequence of this 219 amino acid protein (Mr = 28,500 kDa) has been determined using conventional protein sequencing techniques (Sakamoto et al, 1987) and has also been deduced from cDNA clones isolated from man (Hayasaka et al, 1991) and many animal species including rat (Lemke and Axel, 1985), chicken (Barbu, 1990), and shark (Saavedra et al, 1989). Several features of the primary structure of P0 have subsequently been shown to be related to its localization and function in the myelin membrane.

The first 28 amino acids encoded by the open reading frame of the P0 cDNA do not appear in the mature P0 protein (Lemke and Axel, 1985). This domain comprises a cleavable signal sequence which is necessary for directing P0 to the rough ER and Golgi apparatus for glycosylation (Poduslo, 1984), translocation, and its oriented insertion into the myelin membrane (Lemke and Axel, 1985; D'Urso et al, 1990). Hydrophobicity profiles show that P0 is an integral membrane protein, with a single transmembrane domain (Lemke and Axel, 1985). This divides the protein into an N-terminal extracellular domain and a C-terminal cytoplasmic domain. Each domain possess distinct physical properties. The extracellular domain contains the canonical sequence for N-linked glycosylation (Asn-Gly-Thr), which occurs only once in the deduced P0 structure. Indeed, P0 has been shown to be glycosylated at a only a single position via N-linked attachment, and also, that the attached oligosaccharide is extracellularly positioned (Wood and McLaughlin, 1975; Peterson and Grevner, 1978; Ishaque et al, 1980). Interestingly, the oligosaccharide chain bears the carbohydrate epitopes L2 and HNK-1 (Lemke and Axel, 1985). These epitopes are commonly associated with 'self-adhesive' molecules such as L1, neural cell adhesion molecule (NCAM), and myelin-

associated glycoprotein (MAG) (Edelman, 1983; Ueymura et al, 1987), and it has been suggested that these glycans may mediate homophilic adhesive interactions between P0 molecules on adjacent extracellular myelin membrane faces (Lemke and Axel, 1985; Filbin et al, 1990; Filbin and Tennekoon, 1991).

Further evidence of the adhesive nature of P0 is provided by the sequence of the extracellular domain itself, which exhibits significant similarity to the immunoglobulin (Ig) domain of members of the immunoglobulin superfamily (Lai et al, 1987; Lemke et al, 1988). These proteins are known to mediate a variety of homotypic and heterotypic interactions in the nervous system (Barclay et al, 1987) including cell migration, axonal guidance and myelination (see Salzer and Colman, 1989). Direct evidence for P0 mediated membrane adhesion has been provided by transfection studies involving expressing P0 in HeLa cells (D'Urso et al, 1990; Filbin et al, 1990). The cells form unusual aggregates, coincident with concentration of P0 at the points of cell-cell contact as demonstrated by immunofluorescence analysis.

Of the 69 residues comprising the P0 cytoplasmic domain, 21 are basic and only 6 are acidic. This portion of the molecule therefore carries a very strong net positive charge. With the sequestering of negatively charged lipids (phosphatidylserine) to the cytoplasmic face of the apposed myelin bilayer (Norton and Cammer, 1984), it is highly likely that P0 will interact electrostatically with these lipids (Ding and Brunden, 1995; Wong and Filbin, 1996). Certainly, when P0 is isolated from myelin it is complexed with a set of very tightly bound acidic lipids (Ishaque et al, 1980), particularly phosphatidylserine. Furthermore, phosphorylation of serine residues in the cytoplasmic domain of P0 by protein kinase C (which reduces the overall positive charge of the domain), has been shown to prevent compaction (Suzuki et al, 1990; Hilmi et al, 1995; Rowe-Rendlemann and Eichberg, 1994; Eichberg and Iyer, 1996). It has been postulated that the degree to which P0 is phosphorylated at its C-terminus regulates the strength of electrostatic interaction between P0 and the anionic phospholipids, with progressive diminishing

of P0 phosphorylation resulting in greater myelin compaction. Indeed, developmental dephosphorylation of P0 is known to be concomitant with formation of the major dense line and the maturation of myelin (Suzuki et al, 1990; Hilmi et al, 1995; Rowe-Rendlemann and Eichberg, 1994; Eichberg and Iyer, 1996). However, although this type of electrostatic lipid-protein interaction seems very straightforward, it is possible that apposed P0 cytoplasmic domains connect intracellular membrane surfaces with each other via protein-protein interactions.

Indirect evidence to further support the role of P0 in the maintenance of the major dense line is given by the myelin basic protein (MBP)-deficient *shiverer* mouse (see below). This mutant is characterized by markedly diminished myelination of axons in the CNS (Bird et al, 1978). Where CNS myelin is formed, the major dense line is absent. In contrast, PNS myelin is fully elaborated, has a compact structure with normal periodicity, and is functionally normal (Ganser and Kirschner, 1980). These findings have led to the suggestion that MBPs are involved in compaction of CNS myelin at apposed cytoplasmic faces and that some functionally related component of PNS myelin, not present in the CNS serves this function in the periphery. This component is likely to be P0, given that it is the only abundant protein remaining in isolated PNS myelin from shiverer (Mikoshiba et al, 1981), it localizes at the cytoplasmic surface of the bilayer, both P0 and MBP contain many basic amino acid residues that have been proposed to interact with the phospholipids of the opposing cytoplasmic faces of Schwann cell membranes (Ding and Brunden, 1994), and of course, it is absent from the CNS.

The gene for P0 has been isolated from both rat (Lemke et al, 1988) and mouse (You et al, 1991) and consists of 6 exons distributed over 7 kb (Figure 7). The gene, which is located on mouse chromosome 1 (Kuhn et al, 1991; You et al, 1991) does not show linkage with any known neurological mouse mutation. However, a complete loss-of-function mutation has been engineered by homologous recombination (Giese et al, 1992). These knockout mice develop a

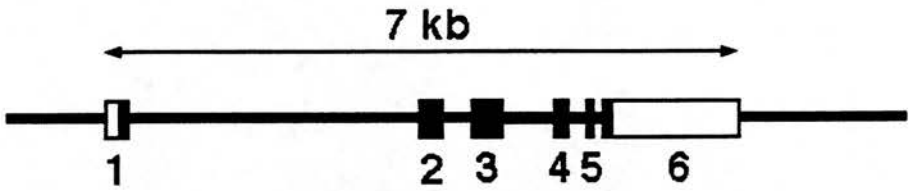


Figure 7. Gene structure of the major myelin protein, P0. Protein coding exons are represented by filled boxes. Unfilled exons correspond to untranslated sequence. After Lemke et al (1988).

dysmyelinating peripheral neuropathy, characterized by poor motor coordination, tremors, and occasional convulsions, all of which develop after the third post-natal week (Giese et al, 1992). Most larger diameter (>1 mm) peripheral axons are surrounded by multiple loops of membrane that remain uncompacted at what (in normal myelin) would be the intraperiod lines. This failure to achieve intraperiod compaction in the absence of P0 gives further support to the hypothesis that the Ig-related extracellular domain of P0 does indeed function as an adhesion protein during myelin spiralling. Importantly, the absence of P0 does not prevent Schwann cells from segregating axon bundles or from assuming a 1:1 relationship with the larger diameter axons or from initiating spiral formation.

Recently, double knockout mutant mice lacking both MBP and P0 have been created from matings of the above mice with *shiverer* (Martini et al, 1995). In these animals both the major dense line and the intraperiod line are lost. As the major dense line is retained in the P0 knockout alone (Giese et al, 1992) it would appear that MBP is involved or can substitute for P0 in compaction of this structure, much as P0 can substitute for MBP in *shiverer* (Ganser and Kirschner, 1980; Mikoshiba et al, 1981). Since MBP does not have an extracellular domain it is unsurprising that it cannot replace the cell-surface related function of P0 relating to compaction of the opposing extracellular faces (Giese et al , 1992; Martini et al, 1995).

Overall therefore, P0 is bifunctional within the myelin membrane, mediating the association of both apposed extracellular and cytoplasmic membrane surfaces by a different molecular mechanism in each case.

(b) Myelin Basic Proteins (MBPs)

The MBPs are a family of small (14 - 23 kDa), membrane-associated proteins (de Ferra et al, 1985; Takahashi et al, 1985; Newman et al, 1987; Kitamura et al, 1990; Aruga et al, 1991). In the PNS, this group can make up almost a fifth of all myelin proteins (Greenfield et al, 1980; Lees and Brostoff, 1984). An identical complement of MBPs are expressed in the CNS (Greenfield et al, 1982), although here they often comprise more than a third of the total myelin proteins.

As their name implies, the MBPs have an unusually high percentage of basic residues (>20%) (Lees and Brostoff, 1984; Smith, 1992). Therefore, like the cytoplasmic tail of P0, these proteins carry a very strong overall positive charge. As a result, the MBPs show a high affinity for acidic lipids within the myelin membrane, such as the sulphatides and the gangliosides (Ong and Yu, 1984; Maggio and Yu, 1992; Mendz, 1992). Given the localization of the MBPs to the cytoplasmic apposition (Lees and Brostof, 1984), and the predominance of acidic lipids on the cytoplasmic face of the myelin bilayer (Norton and Cammer, 1984), it is highly likely that electrostatic interactions between these two elements become involved in myelin compaction, bringing about the fusion of apposed cytoplasmic surfaces to form the major dense line (Lees and Brostof, 1984; Smith, 1992). This is further borne out by the finding that the *in vivo* phosphorylation of MBPs (which decreases their net positive charge) effectively destabilizes compact myelin lamellae structure (Diebler et al, 1990; Martenson et al, 1983; Kobayashi, 1984). The regulation of MBP phosphorylation and dephosphorylation may clearly play a pivotal role in the stabilization of the myelin structure.

The MBP gene consists of 7 exons distributed over 32 kb (Takahashi et al 1985; de Ferra et al 1985; Kimura et al, 1985). In mouse and human, the gene maps to chromosome 18 (Roach et al, 1985; Sparkes et al, 1987). The rat MBP gene maps to chromosome 1 (Koizumi et al, 1991). At least six transcripts are expressed from this gene in the mouse through alternative splicing of three exons (Roach et al, 1983;

Zeller et al, 1984; de Ferra et al, 1985; Takahashi et al, 1985; Newman et al, 1987; Kitamura et al, 1990; Aruga et al, 1991) (Figure 8). Interestingly, this "classic" MBP gene has recently been found to be part of a much larger genetic locus, called the golli-mbp gene complex (for gene in the oligodendrocyte lineage) (Campagnoni et al, 1993; Pribyl et al, 1993). This locus exceeds 100 kb in length and contains two promoters, one of which gives rise to the MBP mRNAs and another that produces at least 3 alternatively spliced golli mRNAs (Figure 8). These MBP-like isoforms are expressed widely outside the nervous system and may serve functions completely unrelated to myelination (Campagnoni et al, 1993; Pribyl et al, 1993; Landry et al, 1996).

MBP isoforms are heterogenous, not only at the level of their primary structure but also as a result of numerous posttranslational modifications including methylation, glycosylation, deamidation and N-acylation (Toews and Morell, 1987; Smith, 1992) which indicates that a single, straightforward role for the MBPs is unlikely. However, the demonstration that mice displaying the *shiverer* phenotype (characterized by a total lack of myelin-associated MBP isoforms and a resulting absence of CNS myelin [Bird et al, 1978]) can be 'rescued' by the expression of an MBP minigene encoding only the smallest MBP protein (14 kDa) (Kimura et al, 1989), casts some doubt on the necessity for this heterogeneity.

(c) Myelin Protein 2 (P2)

Myelin protein 2 (P2) is a small (14 kDa) extrinsic membrane protein (Morell et al, 1989). In the mouse and rat, P2 comprises less than 1% of the total PNS myelin protein content; in the human PNS this value is approximately 14% (Greenfield et al, 1973). Although it is most abundant in the PNS, P2 has been detected in CNS myelin, where it constitutes <1% of the total protein content (Martenson and Uyemura, 1992).

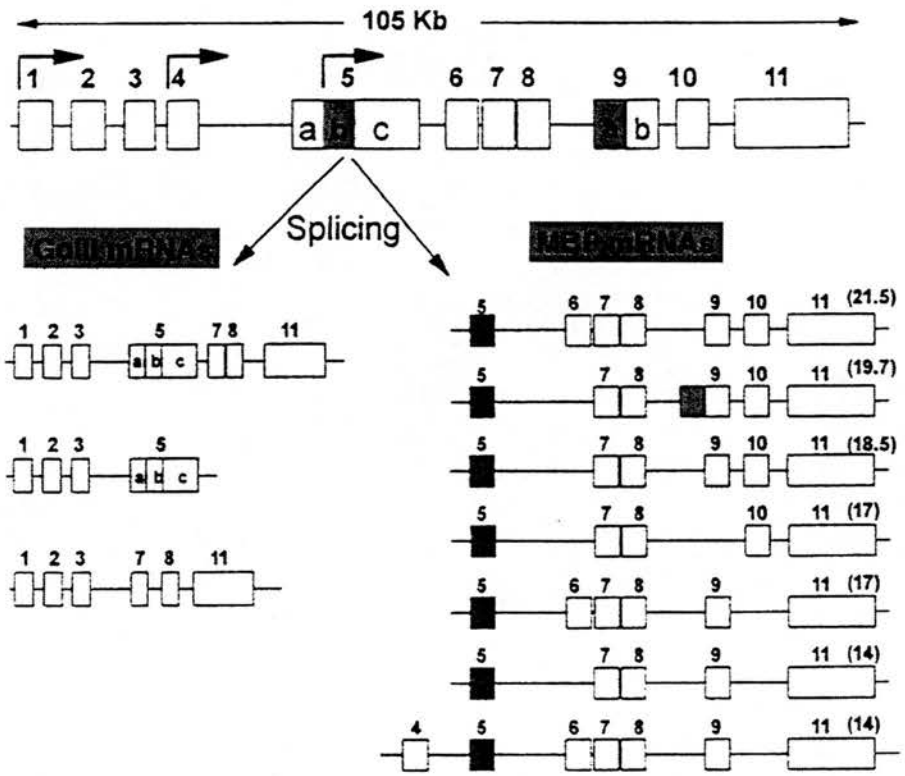


Figure 8. Structure and splicing pattern of the *Golli-mbp* gene. Exons 5-11 encompass the 'classic' MBP gene whereas exons 1-4 are Golli. Bracketed numbers after mRNAs indicate the molecular weight of the translated product. From Campagnoni (1995)

P2 protein has been purified from various species, including cow (Kitamura et al, 1980), rabbit (Isaque et al, 1982), and human (Suzuki et al, 1982). Its deduced amino acid sequence shows a striking homology (40-70%) to a large family of lipid-binding proteins that includes cellular retinol binding proteins, retinoic acid-binding proteins (Crabb and Saari, 1981; Eriksson et al, 1981) and fatty acid-binding proteins (FABPs) (Veerkamp et al, 1981). The related finding that purified bovine P2 can bind oleic acid, retinoic acid and retinol (Uyemura et al, 1984) has therefore prompted the suggestion that the P2 protein is a member of the FABP family with a specific function involving the transport of fatty acids to the myelin membrane and/or the metabolism of myelin lipids (Morell et al, 1989; Narayanan et al, 1988; Martenson and Uyemura, 1992).

The structure of the P2 gene has been determined in the mouse (Narayanan et al, 1991) and in humans (Hayasaka et al, 1993) (Figure 9). In each case the structure is similar and consists of four exons. However, due to a more lengthy intron 1 within the human gene, these 4 exons are distributed over 8 kb in humans compared to only 4.5 kb in the mouse. Interestingly, the mouse P2 gene structure is significantly similar to the gene structures of members of the FABP family of proteins (Narayanan et al, 1991). This finding provides further evidence to support the hypothesis that P2 is a fatty acid-binding protein and indeed, is a *bona fide* member of this family.

(d) Peripheral Myelin Protein 22 (PMP22)

This protein (or its corresponding cDNA) has been identified on at least five separate occasions and has been given a similar number of different names: (1) PASII (periodic-acid-Schiff protein II) (Kitamura et al, 1976); (2) gas3 (growth arrest specific gene 3) (Manfioletti et al, 1990); (3) CD25 (crush denervated cDNA 25) (Spreyer et al, 1991); (4) SR13 (sciatic nerve regeneration clone 13) (Welcher et al, 1991); and finally (5) PMP22 (peripheral myelin protein 22) (Snipes et al, 1992).

Figure 9. Structural organization of the human P2 gene. Exons are shown as boxes and numbered. Solid black boxes and white boxes indicate protein coding sequences and and untranslated sequences, respectively. From Hayasaka et al (1993)

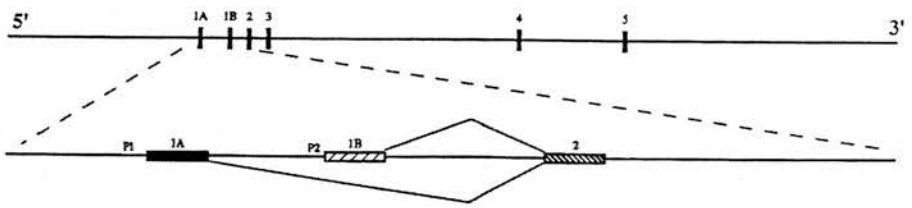


Figure 10. Genomic organisation of the human PMP22 gene. The proposed alternative splicing that results in two different PMP22 transcripts is schematically indicated at the bottom. P1 and P2 refer to the location of promoter 1 and promoter 2. Schwann cell specific expression is driven from promoter 1. From Suter et al (1994).

Most abundant in the PNS where it comprises approximately 5-10% of the total protein of isolated myelin (Kitamura et al, 1976; Snipes et al, 1992; Pareek et al, 1993) PMP22 has become the definitive label for this protein (Snipes et al, 1992). The expression of PMP22 is not restricted to PNS myelin however, and indeed, the multiple separate identifications of this protein reflect the wide expression of PMP22 mRNA within the CNS also and in many non-neural tissues like lung, gut, and heart (Manfioletti et al, 1990; Spreyer et al, 1991; Welcher et al, 1991; Patel et al, 1992).

The open reading frame of the PMP22 mRNA predicts a protein with a molecular weight of approximately 17,000 kDa (Suter et al, 1992). However, by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), PMP22 is seen to migrate at 22 kDa (hence the name). This discrepancy has been shown to be due to N-glycosylation at a single site within the protein (Manfioletti et al, 1990; Spreyer et al, 1991; Welcher et al, 1991; Pareek et al, 1993). From hydrophobicity analyses, the deduced primary sequence of PMP22 indicates that it is a tetraspan protein, traversing the myelin membrane four times (Spreyer et al, 1991). In contrast to P0, a 26 amino acid signal sequence which directs this insertion of the protein into the myelin membrane is not cleaved and is therefore present in the mature PMP22 protein (Manfioletti et al, 1990).

Given that PMP22 exhibits no sequence homology to any previously identified protein it has proved difficult to define its role within PNS myelin. Structurally, PMP22 is remarkably similar to PLP, the major structural protein of CNS myelin (Braun et al, 1984). PLP is believed to play a role in the formation and maintenance of the intraperiod line of CNS myelin (Braun, 1984; Hudson et al, 1987) and it is possible that PMP22 functions in a similar manner in the PNS. The finding that PMP22 of the cat possess the HNK-1 carbohydrate epitope generally expressed by adhesion proteins (Hammer et al, 1993) further suggests an involvement in membrane-membrane interactions. Other speculations as to the role of PMP22 have

included that it acts as an ion channel or pore (Manfioletti et al, 1990), but as yet there are no direct experiments that address this possibility.

The structure of the human *PMP22* gene has been characterized (Suter et al, 1994) (Figure 10). Interestingly, the transcription of the gene is regulated by two promoters that are located immediately upstream of two alternatively spliced 5'-non coding exons (exons 1A and 1B). The polypeptides produced from these two distinct mRNAs are identical (Suter et al, 1994). However, transcripts containing exon 1A are preferentially expressed in myelinating Schwann cells, while transcripts containing exon 1B are preferentially expressed in tissues that do not form peripheral myelin (Suter et al, 1994). It is likely that the promoter governing expression of PMP22 in Schwann cells contains elements which respond to glial-specific transcription factor(s).

The gene for PMP22 maps to mouse chromosome 11 (Suter et al, 1992), within close proximity to the locus of the hypomyelinating PNS neuropathies *Trembler* (*Tr*) and *Trembler-j* (*Tr^j*) (Davisson and Roderick, 1978; Falconer, 1951; Suter et al, 1992b). Mice homozygous and heterozygous for these dominant alleles display peripheral nerve development that is blocked at the promyelinating stage, with Schwann cells singling out individual axons, but failing to proceed to the next step of elaborating myelin (Henry and Sidman, 1988). Furthermore, Schwann cell proliferation continues into adulthood (Aguayo et al, 1977; Henry and Sidman, 1988). Suter and coworkers (1992) have shown that in both Tr and Tr^j mice, PMP22 exhibits point mutations: the mutation in Tr is within the fourth putative transmembrane domain, replacing a glycine residue with an aspartic acid residue (Suter et al, 1992) and in Tr^j it is within the first putative transmembrane domain, replacing a leucine with a proline. Whether these mutant PMP22s, which are presumably misfolded, are correctly incorporated into membrane is unknown, and it remains to be determined what cellular mechanisms prevent myelination.

The continued proliferation of Schwann cells in *trembler* mice is particularly interesting given that PMP22 was previously identified as gas3, a growth-arrest specific protein whose expression has been shown to be negatively correlated with cellular growth (Manfioletti et al, 1990; Suter et al, 1992). It has been suggested therefore that PMP22/gas3 may play a dual role in myelination: at later stages it is involved in the maintenance of the myelin structure and in the early stages it controls Schwann cell proliferation and progression from a promyelinating phenotype.

1.4.3 Minor Constituents of PNS Myelin

Although the vast majority of the protein content of PNS myelin comprises these major proteins, the remaining 1% contains a multitude of minor proteins, splice products, and posttranslationally modified proteins. Such proteins are far less involved in maintaining the compact myelin structure. From their expression and localization patterns it is believed that these proteins perform many of the complex interactions required during the myelination process itself e.g. interacting with the cytoskeleton to drive ensheathment, transducing signals of axonal contact, guiding the membrane tongue as it spirals around the axon, lipid synthesis etc.

(a) Myelin-Associated Glycoprotein (MAG)

In the PNS, MAG comprises only about 0.1% of the total myelin protein content (Figlewicz et al, 1981). However, this belies the fact that MAG is highly concentrated in several areas of the myelinating Schwann cell, including the periaxonal regions, Schmidt-Lantermann incisures, paranodal loops, and the inner and outer mesaxons (Schober et al, 1981; Sternberger et al, 1979; Trapp and Quarles, 1982). MAG is absent from compact myelin. A common characteristic of these regions is that they are filled with Schwann cell cytoplasm (ie they are uncompacted) (see section 1.3.3). Indeed, there is a strict correlation between the

presence of MAG and the periodicity of these membranes (Trapp and Quarles, 1982). It has been suggested therefore that MAG plays a role in maintaining these spaces by preventing compaction (Trapp and Quarles, 1982; Trapp et al, 1984). The necessity for retaining cytoplasm-filled regions through the myelin sheath remains unclear, but given that turnover of the sheath components is known to occur (Smith, 1968; Benjamins and Smith, 1984; Miller et al, 1977), it possible that these regions act as channels for transport of the necessary materials throughout the compact myelin structure (Hirano, 1982).

During SDS-PAGE of PNS myelin proteins, MAG is seen to migrate as a characteristically broad band of approximately 100 kDa (Barbarash et al, 1981). When enzymatically deglycosylated, MAG resolves as two isoforms of 72 and 67 kDa, the latter of which is by far the most abundant (Frail et al, 1985; Tropak et al, 1988). Both MAG isoforms are also found in CNS myelin but interestingly, the larger 72 kDa isoform is present at much higher levels (Salzer et al, 1990; Quarles et al, 1992) and indeed is the predominant isoform during the most active period of myelination (Tropak et al, 1988).

The primary structure of each isoform has been deduced from cDNA clones isolated from a number of species including human (Sato et al, 1989; Spagnol et al, 1989), rat (Lai et al, 1987; Salzer et al, 1987) and mouse (Arquint et al, 1987). Both isoforms share a common 17 amino acid N-terminal signal sequence, followed by a large extracellular N-terminal region, and a 23 amino acid membrane spanning domain. It is in the sequence of their respective C-terminal cytoplasmic tails that the isoforms show divergence, with the final 53 amino acids of the large MAG isoform (named L-MAG) being replaced by a different sequence of 9 amino acids in the small MAG isoform (named S-MAG) (Lai et al, 1987; Salzer et al, 1987). From the deduced structure of the MAG gene, it clear that these two isoforms arise through alternative splicing.

In both the rat and mouse, the *MAG* gene contains 13 exons and spans approximately 16 kb (Lai et al, 1987; Nakano et al, 1991) (Figure 11). As shown, the mRNA coding for L-MAG contains all exons except exon 12, whereas the mRNA coding for S-MAG consists of all exons except exon 2 (Lai et al, 1987). For L-MAG, the termination codon is present in exon 13. The presence of exon 12 in the S-MAG mRNA introduces an earlier in-frame termination codon, and results in the formation of a smaller protein. The presence of exon 2 does not affect the polypeptide produced from the L-MAG mRNA as it is part of the 5' untranslated region and indeed, alternative splicing of this exon seems to be regulated independently of the splicing of exon 12 (Lai et al, 1987; Tropak et al, 1988). This is supported by the finding of a MAG mRNA without either exon 2 or 12 (Fujita et al, 1989) (Figure 11).

It is reasonable to assume that the different cytoplasmic tails of the two isoforms bestow different physical (and perhaps functional?) properties and certainly, the C-terminal tail of L-MAG is known to be phosphorylated to a greater extent than the C-terminal tail of S-MAG (Edwards et al 1988; Lai et al, 1987; Salzer et al, 1987; Arquint et al, 1987; Fujita et al, 1989; Agarawal et al, 1990). As MAG has been postulated to interact with the cytoskeletal elements of myelin-forming cells such as actin and spectrin (Trapp et al, 1984; Salzer et al, 1987; Quarles et al, 1992), it is possible that the phosphorylation state of each isoform may modulate its interactions with these elements during different stages of the myelination process. Furthermore, the cytoplasmic tail of L-MAG also contains a unique tyrosine residue that is surrounded by a sequence highly homologous to an autophosphorylation site present in the cytoplasmic domain of the epidermal growth factor receptor and which is suggested to play a role in signal transduction (Arquint et al, 1987; Salzer et al, 1987; Lai et al, 1987). However, although these finding may be of importance to myelination in the CNS where both L-MAG and S-MAG reach comparable levels, their relevance to PNS myelination is questionable

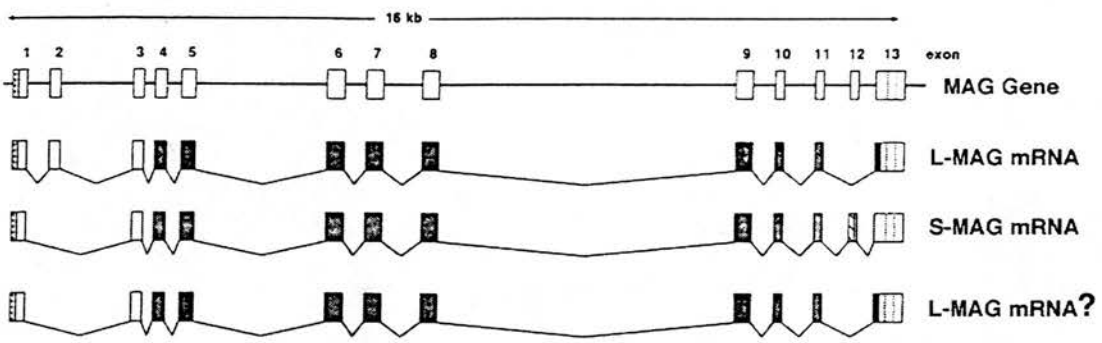


Figure 11. Structure and alternative splicing of the rat myelin-associated glycoprotein (MAG) gene. Exon sizes are exaggerated and not proportional. Three alternatively spliced products are shown. Splicing of exons 2 and 12 is not coordinately regulated. After Sutcliffe (1987) and Fujita et al (1989).

given that L-MAG is an extremely minor component. To define a precise role for the MAGs in myelination, the greatest attention has therefore been focused on the common extracellular domain.

Detailed analysis of the sequence of the MAG extracellular domain has identified 5 sub-domains with varying degrees of homology to each other. Like P0, each sub-domain also shares significant similarity with the immunoglobulin (Ig) domain of members of the immunoglobulin superfamily (Arquint et al, 1987; Lai et al, 1987; Salzer et al, 1987). As previously stated, these proteins are known to mediate cell-cell interactions in the nervous system. Given the localization of MAG to the periaxonal region of the Schwann cell, it has been suggested that MAG must therefore be involved in recognition and/or adhesion between the myelinating glial cells and the axons destined to be myelinated (Arquint et al, 1987; Lai et al, 1987; Salzer et al, 1987).

The adhesive nature of the extracellular domain of MAG is further supported by its complement of carbohydrate moieties, which contains the L2/HNK-1 epitope (Sutcliffe et al, 1983; Salzer et al, 1987; Lai et al, 1987). This epitope is shared by a number of nervous system cell adhesion molecules including N-CAM, L1, contactin (Milner et al, 1990; Kruse et al, 1984), and is believed to be itself a ligand of adhesion. Direct experimental evidence of the adhesive nature of MAG with regards to neurons has been provided by a number of groups, including Poltorak and co-workers (1987) who demonstrated that purified MAG reconstituted in liposomes can bind axons.

The most intriguing finding to date as regards MAG function has been gained from the analysis of null MAG mutant mice (Montag et al, 1994; Li et al, 1994). Although these mice do form compact PNS myelin, the organization of the periaxonal region is partially impaired. Importantly, these animals display significant peripheral nerve abnormalities indicative of degeneration by the time they are 8- to 10-months old (Fruttiger et al, 1995). These findings seem to

suggest that MAG is not critical for myelin formation in the PNS but rather is necessary for maintenance of the cytoplasmic collar and periaxonal space which may be essential for the prolonged maintenance of normal interactions between axons and myelinating SC.

(b) 2',3'- cyclic nucleotide-3'-phosphodiesterase (CNPase)

The early view held of myelin was that of a metabolically inert material. However, since the discovery of CNPase enzyme in purified myelin preparations (Kurihara and Tsukada, 1967) this belief has been refuted. Forming 0.5-1% of PNS myelin proteins and 2-5% of myelin proteins from the CNS (Vogel and Thompson, 1988; Sprinkle, 1989; Tsukada and Kurihara, 1992), CNPase is by far the most abundant of more than 40 myelin-associated enzymes that have since been found. CNPase is also detectable at very low levels in cells outside of the nervous system, most notably in lymphoid tissue, erythrocytes, and platelets (Bernier et al, 1987; Sprinkle et al, 1985). CNPase is therefore not strictly myelin-specific although, within the nervous system, it is found only in myelin-forming glia. Indeed, CNPase is one of the first reliable markers of these cells (Kurihara and Tsukada, 1968; Olafson et al, 1969; Kurihara and Tsukada, 1969), becoming concentrated in the periaxonal region, paranodal loops and the inner mesaxon during myelination (Sprinkle et al, 1989; Braun et al, 1990). CNPase is not observed in compact myelin (Sprinkle, 1989; Braun et al, 1990; Tsukada and Kurihara, 1992).

In terms of its enzymatic activity, CNPase hydrolyses 2', 3'-cyclic nucleotides to generate 2' nucleotides (Sprinkle, 1989; Tsukada and Kurihara, 1992). However, as no 2',3'-cyclic nucleotides or 2'-nucleotides have been found in myelin, it has been postulated that the enzymatic activity of CNPase is vestigial and irrelevant and that the protein's true function is still to be discovered (Norton and Cammer, 1984; Braun et al, 1990; Tsukada and Kurihara, 1992).

SDS-PAGE of purified CNPase under reducing conditions resolves two bands, designated CNP1 and CNP2. These polypeptides show apparent molecular weights between 42 and 49 kDa respectively, depending upon the species studied, with rat CNPase having the highest molecular weights (47 kDa and 49 kDa) and human CNPase the lowest (42 kDa and 45 kDa) (Muller at al, 1981; Muller et al, 1982; Tsukada and Kurihara, 1992). It is believed that CNP1 and CNP2 are subunits of the native CNPase enzyme since electrophoresis under non-denaturing conditions resolves a single high molecular weight band only.

Analysis of cDNAs encoding each CNPase isoform has demonstrated that CNP1 differs from CNP2 by the presence of an additional 20 amino acid residues at the amino terminal end of CNP2 (Kurihara et al, 1990; Gravel et al, 1994). The sequence of each isoform reveals no significant regions of hydrophobicity that could serve as a transmembrane domain, confirming a cytoplasmic localization. Interestingly, a high concentration of dicarboxylic acid residues suggests that the proteins must be amidated (Kurihara et al, 1981). Like MBP and P0 therefore, CNPase has strongly charged groups available for interactions with acidic lipids of the myelin membrane bilayer (Lees and Brostoff, 1984). The relevance of this finding to the role of CNPase is unknown.

A number of functions have been postulated for CNPase, including a role in tRNA ligase reactions, cell adhesion, or kinase reactions (reviewed by Vogel and Thompson, 1988; Sprinkle, 1989; Tsukada and Kurihara, 1992) but none are conclusive. The most compelling evidence for CNPase function involves the isoprenylation of CNP1 at a C-terminal cys-X-X-X-COOH site (Braun et al, 1991). Other proteins known to be isoprenylated at the C-terminus, such as ras proteins and some G protein subunits, are associated with signal transduction pathways (Braun et al, 1991). Together with the presence of potential nucleotide-binding domains (Sprinkle, 1989), this finding provides evidence for the hypothesis that

CNPase may exert a regulatory influence on myelination by serving as a link between extracellular signals and intracellular effector molecules.

(c) Proteolipid protein (PLP)

Proteolipid protein (PLP) is the major protein component of CNS myelin, accounting for more than 50% of the total protein content (Folch-Pi and Lees, 1951; Agarawal et al, 1972; Agarawal and Hartman, 1980). This extremely hydrophobic, integral membrane protein (Braun et al, 1984) is believed to play a role in the formation and maintenance of the intraperiod line of CNS myelin (Braun, 1984; Hudson et al, 1987). To a certain extent therefore, PLP is considered to be the CNS equivalent of P0 (Braun, 1984; Lemke and Axel, 1985). Like P0, PLP was considered highly cell-type specific, in as much as it was detectable solely in oligodendrocytes (whereas P0 is expressed solely in myelinating Schwann cells). However, a number of studies have recently shown that PLP mRNA is transcribed in small but significant quantities in Schwann cells (Puckett et al, 1987; Agarawal and Agarawal, 1991) and that PLP protein is actively translated, subjected to the same post-translational modifications as its CNS counterpart, and is incorporated into compact PNS myelin (Agarawal and Agarawal, 1991; Tetzloff and Bizzozero, 1993).

The role of PLP in the PNS is puzzling, especially when considering the minute amounts of protein present in this tissue compared to CNS myelin. However, the recent discovery of a PLP null mutation in humans which presents as a demyelinating peripheral neuropathy (Garbern et al, 1997) indicates that this role is nevertheless vital.

1.5 HUMAN PERIPHERAL NEUROPATHIES

1.5.1 Myelin Neuropathies

Given the importance of myelin to the normal functioning of the nervous system, in particular rapid impulse conduction, any loss or injury of myelin sheaths is likely to have a deleterious effect. Unfortunately, the complexity of myelin, both in terms of its structure and the developmental programme by which it is elaborated, provides many opportunities for such damage to arise. This is clearly evident from the distressingly high number of human neurological diseases known to be caused by demyelination (the loss of previously formed myelin) or dysmyelination (the failure to elaborate functional myelin) such as multiple sclerosis, the adrenoleukodystrophies, Pelizaeus-Merzbacher disease, Guillain-Barre syndrome, the Charcot-Marie-Tooth neuropathies, Dejerine-Sottas syndrome, and many more (for reviews see Raine, 1984; Allen, 1984; Scriver et al, 1989; Scolding et al, 1994; Suter and Snipes, 1995).

Importantly, many of the above diseases predominantly affect either the oligodendrocyte myelin of the CNS or the Schwann cell myelin of the PNS. For instance, multiple sclerosis (MS) is a demyelinating neuropathy of man which selectively targets only myelin sheaths of the CNS (Martyn, 1991). With more than 80,000 sufferers (1 person in every 1000) in the UK alone, and an estimated 2.5 million worldwide, MS is the most prevalent of these disorders, presenting symptoms which may include spasticity, fatigue, visual problems, and impaired cognitive function, but which all reflect impairment of the CNS. The most common PNS-specific human neuropathy belongs to a genetically heterogenous group of disorders known as the Charcot-Marie-Tooth (CMT) diseases. The most prevalent form, CMT1A, affects approximately 1 in 2500 people (Skre 1974; Vance et al, 1991) which is lower than the prevalence of MS. Symptoms include progressive

muscular atrophy and weakness, reduced nerve conduction velocities, and segmental demyelination and remyelination.

1.5.2 Origins

From the overlapping but not identical composition of myelin elaborated by Schwann cells and oligodendrocytes, it is probable that it is the unique proteins and epitopes which are responsible for the selective pathology of these diseases. Indeed, from linkage studies and gene sequence analysis, mutations of many of these unique components have shown strong associations with myelin dysfunction (Nave, 1994; Griffiths, 1996; Scherer, 1997).

As discussed in section 1.4, Schwann cells express a number of proteins which are predominantly found in PNS myelin including P0, PMP22 and P2. To a greater or lesser extent, mutations of each of these proteins are believed to be involved in the most common peripheral neuropathies:

(a) PMP22

As discussed in section 1.4.2(d), point mutations of the murine *PMP22* gene have been implicated in the *trembler* and *trembler-j* phenotypes (Suter et al, 1992). The region of mouse chromosome 11 that contains these mutations is syntenic with a region of the proximal short arm of human chromosome 17 (17p11.2) which interestingly, has been identified by linkage analysis as a locus for CMT1A (Vance et al, 1989; Hoogendijk et al, 1991). Furthermore, it has been shown that the human *PMP22* locus is similarly located in this region and that it is mutated in patients with CMT1A (Matsunami et al, 1992; Patel et al, 1992; Timmerman et al, 1992; Valentijn et al, 1992a). For most, this mutation takes the form of a large (approximately 1.5 Mb) duplication. Importantly, the *PMP22* gene is duplicated in its entirety within this region and does not appear to be interrupted or inactivated (Chance et al, 1992; Lupski et al, 1992; Hanemann et al, 1994). This suggests that

the CMT1A phenotype can arise through over expression of PMP22 protein (Vallat et al, 1996). Moreover, homozygosity of the 1.5 Mb duplication (generating four copies of the PMP22 gene) causes a much more severe demyelinating neuropathy called Dejerine-Sottas syndrome (DSS) (Scherer et al, 1997).

As well as PMP22 overexpression, CMT1A has been attributed in some families to point mutations in the *PMP22* gene (Roa et al, 1993; Valentijn et al, 1992b, 1993; Gabreels-Festern et al, 1995; Marrosu et al, 1997; Navon et al, 1996), some of which correspond precisely to those of *trembler* (Valentijn et al, 1992b). Certain other mutations of the PMP22 gene have been implicated in another peripheral disease, hereditary neuropathy with liability to pressure palsies (HNPP). This recurrent demyelinating condition is believed to be associated with a deletion of the same region of chromosome 17 that is duplicated in CMT1A patients (Chance et al, 1993; Nicholson et al, 1994; Vallat et al, 1996; Schenone et al, 1997). Further support for this association is demonstrated by a *PMP22*-null knockout mouse which displays a remarkably similar phenotype to HNPP (Adlkofer et al, 1997a; Adlkofer et al, 1997b).

Thus, different alterations of the *PMP22* gene, including point mutations, duplications and deletions may give rise to distinct hereditary peripheral neuropathies

(b) Protein zero (P0)

Although no naturally occurring mutations or neuropathies associated with P0 have been identified in animals, nearly 30 mutations of the human P0 gene locus (1q21-23) have been described (Warner et al, 1996). Moreover, this locus shows linkage to Charcot-Marie-Tooth type 1B (CMT1B) (Bird et al, 1982; Lebo et al, 1991; Hayasaka et al, 1993; Kulkens et al, 1993; Suter and Snipes, 1995). Interestingly, mutations of P0 have also been associated with Dejerine-Sottas syndrome, not just PMP22 (see above).

(c) Protein 2 (P2)

The genetic locus for P2 has been mapped to the long arm of human chromosome 8 (8q21.3-q22.1) (Hayasaka et al, 1993). This region is closely linked to a marker for the autosomal recessive Charcot-Marie-Tooth disease type 4A (CMT4A) (Ben Othmane et al, 1993). The possibility that this peripheral neuropathy is due to a defect in the P2 gene has therefore been raised, but not confirmed.

(d) Connexin32 (Cx32)

The recent discovery of this gap junction protein within regions of non-compact myelin (Schmidt-Lanterman incisures and paranodal loops) (Scherer et al, 1995) has proved of great interest following the mapping of its locus to Xq13, a region which shows association with X-linked Charcot-Marie-Tooth (CMTX). Numerous studies have demonstrated a variety of mutations in the *Cx32* gene of people with this disease (e.g. Bergoffen et al, 1994; Fairweather et al, 1994; Ionesescu et al, 1995; Bone et al, 1995), affecting all portions of the Cx32 protein.

As typical gap junctions are not seen in myelinating Schwann cells the presence of Cx32 is intriguing. Given that null mutant mice for this gene display a persistent peripheral demyelinating neuropathy (Nelles et al, 1996; Anzini et al, 1997) it is becomes obvious that connexin 32 does play an important role within myelinating Schwann cells. Interestingly, the CNS myelin of these knockout animals appears normal . This is surprising as oligodendrocytes also express Cx32. However, oligodendrocytes also express another type of connexin, Cx45 (Kunzelmann et al, 1997) and it has been suggested that this molecule can substitute Cx32 in these mice. Further studies are required to determine the precise role of connexins in myelin-forming cells.

1.5.3 Uncharacterized Neuropathies

Whereas the most common inherited peripheral neuropathies appear to be caused by mutations in the genes for PMP22, P0, P2 and Cx32 there are probably many rarer types associated with other myelin and/or Schwann cell proteins. Indeed, the neuropathies described so far have only implicated mutations in genes encoding the structural proteins of myelin, whereas it is just as likely that mutations of regulatory genes and genes involved in the developmental program of the Schwann cell will show linkage to other myelin diseases. Given the functional information obtained from observing the effects of mutations on the myelin structural genes and the resulting myelin that is formed (if any), the identification of linkages between regulatory genes and myelin structure and function will prove to be invaluable.

1.6 BACKGROUND TO PROPOSED STUDY

1.6.1 Periaxin is a recently described protein of myelinating Schwann cells

Schwann cells undergo major morphological changes during the development of the PNS (Jessen and Mirsky, 1991). From their insinuation into nerve bundles, to establishing the 1:1 relationship with the axon, to the repeated spiral wrapping during the myelination process, all of these events necessarily involve massive alterations in cell shape. Such changes are obviously an essential component of the Schwann cell differentiative programme and it follows that the mechanisms involved in effecting these changes must be vital in driving the development of the PNS. Substantial evidence exists to show that these morphological alterations are triggered by axon-Schwann cell interactions (Gould et al, 1992; Neuberger and De Vries, 1992). However, the precise molecular nature of these interactions, in

particular the mechanisms of signal transduction and the targets of such signals, remain largely unknown.

For any cell, a change in shape requires remodelling of the cell cytoskeleton and its associated proteins. In the case of Schwann cells, these elements would appear to be ideal targets for modulating the developmental signals initiated through axonal contact. It follows that the identification of Schwann cell cytoskeletal and cytoskeleton-associated proteins whose patterns of expression and localization are developmentally regulated during differentiation and myelination could prove to be extremely useful. On this premise, Gillespie and co-workers (1994) have recently reported the molecular cloning of a relatively abundant protein of myelinating Schwann cells which has properties of a cytoskeleton-associated protein based on its insolubility in non-ionic detergent.

From its predominant localization to the periaxonal region of myelin-forming Schwann cells in developing peripheral nerves, this protein has been named periaxin (Gillespie et al, 1994; Scherer et al, 1995). Importantly, two other proteins with a similar mobility by SDS-PAGE as periaxin have previously been isolated from PNS myelin: P170 and Schwann cell associated glycoprotein (SAG) (Shuman et al, 1986, 1988; Dieperink et al, 1992). Based on similar apparent molecular weights, immunohistochemical localizations, and triton X-100 insolubility, it is probable that periaxin, P170 and SAG are one and the same protein (Dieperink, 1992; Gillespie et al, 1994; Scherer et al, 1995).

The primary structure of rat periaxin has been deduced from a full length cDNA clone (Gillespie et al, 1994). Neither the nucleotide nor the deduced amino acid sequence of the encoded 147 kDa periaxin protein is significantly similar to any other sequence in the Genbank or EMBL databases. From hydrophobicity plots, periaxin does not appear to possess any transmembrane domains and is therefore not believed to be an integral membrane protein. However, it does display an interesting domain structure, including a highly basic region near the N-terminus

between amino acids 118 and 196 and an acidic region encompassed by residues 1036-1163 towards the C-terminus (Figure 12A). The most interesting domain, spanning approximately 300 amino acids and located towards the centre of the molecule, displays a remarkable repeating series of a pentapeptide (aliphatic nonpolar:pro:glu or asp: aliphatic nonpolar:variable) and a tripeptide spacer (Figures 12A, B). Given that this domain is believed to be less flexible than the rest of the periaxin molecule (Gillespie et al, 1994), it has been suggested that the repeats serve to extend the protein, thus keeping the (functional?) basic and acidic domains apart. Indeed, this bipolar structure may be indicative of a linking role for periaxin within myelinating Schwann cells, with the domain at one end of the protein connected to the Schwann cell plasma membrane and the domain at the other associated with the cytoskeleton.

The most instructive features of periaxin as regards its function, is its expression which is highly regulated both spatially and temporally in the developing peripheral nervous system of the rat (Gillespie et al, 1994; Scherer et al, 1995). Periaxin is first detected in Schwann cells after they establish a 1:1 relationship with axons and are ready to proceed with myelination (Gillespie et al, 1994; Scherer et al, 1995). As demonstrated by immunofluorescence microscopy, this precedes the expression of the major myelin proteins P0 and MBP and may even precede the very early expression of MAG (Scherer et al, 1995; Martini and Schachner, 1986, 1988; Hahn et al, 1987; Lamperth et al, 1990). Thereafter, the levels of periaxin mRNA and protein closely parallel those of the myelin structural proteins which become maximally expressed during the most active phase of PNS myelination (post-natal days 1 to 15 in the rat) (Webster and Favilla, 1984). Like other myelin-related proteins therefore, the expression of periaxin is tightly controlled by axon-Schwann cell interactions (Scherer and Salzer, 1995).

Within the Schwann cell, periaxin protein is initially localized to the periaxonal membranes, the paranodal loops and Schmidt-Lanterman incisures, and

A.

B.

432	GPEVK	APK	531	VPEMK		627	IPDMA	
440	GPEVK	LPK	536	LPDMK	LPK	632	VPDVR	
448	VPEIK	LPK	544	VPEMA		637	LPEVQ	LPK
456	APEAA		549	VPDVH		645	VSELK	LPK
461	IPDVQ		554	LPDIQ	LPK	653	VPEMT	
466	LPEVQ	LPK	562	VPEMK		658	MPDIR	
474	MSDMK	LPK	567	LPDMK	LPK	663	LPEVQ	LPK
482	IPEMA		575	VPEMA		671	VPDIK	
487	VPDVH		580	VPDVR		676	LPEIK	LPK
492	LPEVK	LPK	585	IPEVQ	LPK	684	VPEMA	
500	VPEMK		593	VSEVK	LPK	689	VPDVP	
505	VPEMK	LPK	601	IPDMA		694	LPELQ	LPK
513	IPEMA		606	VPDVR		702		VPQ
518	VPDVH		611	LPELQ	LPK	705	VPDVH	LPK
523	LPDIQ	LPK	619	MSEVK	LPK	713	VPEMK	LPK
						721	VPEAQ	

Figure 12. The domain structure of periaxin. (A) Analysis of the primary structure of periaxin indicates the presence of 3 domains (1) a strongly basic N-terminal domain from 118-196, (2) an acidic C-terminal domain from 1036-1163, and (3) a repeat domain comprising multiple copies of a pentapeptide sequence and tripeptide spacer (Gillespie et al, 1994). (B) Alignment of repeat region residues. The smallest repeating units are a pentapeptide and a tripeptide spacer. The pentapeptide is comprised of the following amino acids: aliphatic nonpolar:pro:glu or asp:aliphatic nonpolar:variable. The underlined sequences substitute serine for proline. Residue numbers are indicated. From Gillespie et al (1994).

is absent from compact myelin (Gillespie et al, 1994). As the myelin sheaths mature, this localization becomes predominantly abaxonal (apposing the basal lamina) (Scherer et al, 1995). This shift in the localization of the protein in the Schwann cell after completion of the spiralization phase of axon ensheathment suggests that periaxin may participate in the membrane protein interactions that are required to stabilize and maintain the mature sheath.

1.6.2 Proposed Study

Of those genes that are typically expressed by myelin-forming Schwann cells, the *periaxin* gene is one of the first to become transcriptionally active (Scherer et al, 1995). The means by which the gene is regulated is therefore of considerable interest in evaluating how the expression of those genes required for initiating axonal ensheathment and myelination is coordinated. Moreover, characterization of the mechanisms and elements involved in determining the tissue-specific expression of this and other genes will be central to our understanding of the processes involved in vertebrate development. Analysis of the regulatory elements of the *periaxin* gene, in particular the upstream promoter, may yield valuable information towards these goals.

The initial aim of this study therefore, was to isolate and characterize the mouse *periaxin* gene so as to provide a firm grounding for future studies on the molecular basis of *periaxin* gene regulation. Furthermore, this information and material would prove to be immensely useful in the generation of knockout mice in an attempt to shed some light on the role of periaxin in myelinating Schwann cells. Thus, this work involves the screening of a genomic library for clones encoding the periaxin gene, followed by detailed structural and sequence analysis, especially of the core promoter. Importantly, although earlier work has been focused on rat periaxin, in this study the emphasis was placed on mouse periaxin to more readily facilitate these future investigations.

Methods

2.1 SCREENING OF GENOMIC LIBRARY

2.1.1 Special reagents and materials

Genomic library

The genomic library screened during this procedure was of mouse strain 129SV (a gift from Dr W. Skarnes, Centre for Genome Research, Edinburgh University); it was constructed in the replacement vector Lambda DASH II (Stratagene; see appendix) by ligation of a partial *Sau*3A1 restriction digest of 129SV genomic DNA into *Bam*H1 cut λ arms and then packaged with Gigapack Gold packaging extracts (Stratagene). The titre of the library was 26,000 plaque forming units (pfu)/μl.

Host bacterium for library phage

NZY broth (Gibco BRL) containing maltose (0.2%) was inoculated with bacterial strain NM675 then incubated at 37°C for 6 h with shaking.

Probe

A full length cDNA corresponding to the 4.6 kb rat periaxin mRNA (pA1BG; Gillespie et al, 1994) was double-digested with the restriction enzymes *Sal*1 and *Xho*1 then electrophoresed on a 1% agarose gel in TAE. The 3 kb fragment (corresponding to nts 1-2992 of the 4.6 kb rat periaxin cDNA) was excised and purified with Qiaex II (Qiagen) according to the manufacturers instructions. The purified fragment was subsequently labelled with [α-^{32}P] dCTP by random priming (Gibco BRL).

2.1.2 Method

Plating of library clones:-

1. Fresh host cell culture (100 μl) and genomic library (2 μl; ~50,000 pfu) were dispensed into 20 sterile bijous.

2. After incubating at 37°C for 10 min to allow phage particles to adsorb to the bacteria, molten (50°C) NZY top agarose (0.7%; 6.5 ml) was then added to the first

bijou, mixed by inversion and immediately poured on a 145 mm plate containing 50 ml of hardened NZY agar (1.5%). The plate was then swirled gently to ensure an even distribution.

3. The above step was subsequently repeated for the other bijous.

4. When the top agarose had hardened (approximately 10 min), the plates were inverted and incubated overnight at 37°C.

Plaque lifts:-

1. Following overnight incubation, the plates were chilled at 4°C for 2 h to prevent the top agarose sticking to the filters during the plaque lifts.

2. Prior to performing the plaque lifts the nitrocellulose filters (137 mm circles; Duralon, Stratagene) were labelled with a corresponding plate letter (A-T), and a small (~ 1 cm) triangular portion carefully cut from their outside edge.

3. The first filter was laid onto the surface of the first plate, taking care not to trap air bubbles. To establish future orientation, 3 holes were punched at asymmetric positions around the filter edge, into the agar, using a 16-gauge needle. The position of these holes and the triangular cut in the filter were then marked onto the underside of the plate with indelible ink.

4. After 2 min, the filter was carefully removed with forceps and placed contact side up on a large sheet of 3MM paper (Whatman) soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min.

5. The filter was then transferred to a fresh sheet of 3MM paper soaked in neutralizing solution (1.5 M NaCl, O.5 M Tris HCl, pH 8.0). After 3 min, the filter was transferred to a fresh sheet of 3MM paper, again soaked in neutralizing solution, and left for a further 3 min.

6. Finally, the filter was rinsed briefly in 2x SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0), allowed to dry, then baked at 80°C for 2 h between sheets of 3MM paper.

7. Steps 3-6 were repeated with the second and subsequent filters and their corresponding plates.

8. All plates were returned to storage at 4°C until required.

Prehybridization of plaque-lift filters:-

1. After briefly wetting with distilled water, the plaque-lift filters were arranged into 10 siliconized glass hybridization tubes (roller bottles; Hybaid).

2. QuikHyb solution (15 ml; Stratagene) was pipetted into each tube, ensuring the filters were fully covered, and the tubes then incubated at 60°C for 30 min (with constant mixing).

Hybridization of plaque-lift filters:-

1. Sheared salmon sperm DNA (100 µl of 10 mg/ml stock; Stratagene) was added to each of 10 microfuge tubes containing 12.5 ng of labelled probe (see special reagents and materials).

2. After boiling for 5 min, the probes were immediately added to the prehybridization solution in each tube, ensuring thorough mixing and filter coverage.

3. Tubes were then incubated for a further 1.5 h at 60°C.

Post-hybridization washes:-

Following the hybridization, filters were washed twice in 2x SSC/ 0.1% SDS (10 min per wash; room temperature) and twice in 0.2x SSC/ 0.1% SDS (30 min per wash; 65°C).

Probe detection:-

1. Filters were arranged in X-ray cassettes (with intensifying screens) and their orientation marks outlined with Indian ink containing [α-^{32}P]dCTP.

2. After carefully overlaying with food wrap (SARAN), the filters were exposed to X-ray film (Fuji RX) overnight at room temperature

3. Autoradiographs were developed using LX24 X-ray developer and Industrex manual fixer (both Kodak).

4. After identifying 'putative' positives from the autoradiographs (19), the films were aligned with the plates using the orientation marks and a plug of agar taken, corresponding to the position of the putative clones.

5. Each plug was then transferred to a separate large microfuge tube containing SM buffer (500 µl; 50 mM Tris HCl pH 7.5, 100 mM NaCl, 8 mM $MgSO_4.7H_2O$) and chloroform (20 µl). Tubes were then incubated at room temperature for a minimum of 6 h to allow phage particles to diffuse from the plugs.

NB. Primary agar plugs were taken using an inverted blue (200-1000 µl) pipette tip, secondary plugs with an inverted yellow (0.5-200 µl) pipette tip and tertiary plugs with a Pasteur pipette.

Titration of primary plugs:-

1. One third (*6*) of the putative clones were selected at random. A 10^{-3} dilution of each (5 µl) was then used to infect fresh host cell culture (100 µl).

2. After incubating at 37°C for 10 min, each titration was then plated with molten (50°C) NZY top agarose (0.7%; 3 ml) on a 90 mm plate containing 15 ml of hardened NZY agar (1.5%).

3. Once the top agarose had hardened, the plates were inverted and incubated overnight at 37°C.

4. Plug titres were subsequently calculated from the resulting numbers of visible plaques. For the un-titred clones the average was used.

Secondary screening of primary clones:-

1. Approximately 1000 pfu from each primary plug were plated on single 90 mm NZY agar plates using fresh host cells (100 µl) and NZY top agarose (3 ml), then incubated overnight at 37°C.

2. A nitrocellulose filter plaque-lift was then taken from each plate as before.

3. Prehybridization, hybridization, washes and probe detection were then carried out, also as before.

4. After orienting the resulting autoradiographs to their corresponding plates, a single positive clone plug was taken from each plate and transferred to a separate microfuge tube containing SM buffer (250 µl) and chloroform (10 µl). Tubes were then incubated at room temperature for 6 h.

5. All secondary plugs (*12*) were subsequently titred (essentially as for the primaries except using 1 µl of 10^{-1} dilutions).

Tertiary screening of secondary clones:-

1. Approximately 100 pfu from each primary plug were plated on single 90 mm NZY agar plates using fresh host cells (100 µl) and NZY top agarose (3 ml), then incubated overnight at 37°C.

2. A nitrocellulose filter plaque-lift was then taken from each plate as before.

3. Prehybridization, hybridization, washes and probe detection were then carried out, also as before.

4. After orienting the resulting autoradiographs to their corresponding plates, a single positive clone plug was taken from each plate and transferred to a separate microfuge tube containing SM buffer (250 µl) and chloroform (10 µl). Tubes were then incubated at room temperature for 6 h.

5. All tertiary plugs (*12*) were subsequently titred (essentially as for the secondaries).

Preparation of a high titre stock of each clone:-

1. Approximately 150,000 pfu from each tertiary plug were plated on single 145 mm NZY agar plates using fresh host cells (300 µl) and NZY top agarose (6.5 ml), then incubated overnight at 37°C.

2. Plates were subsequently flooded with SM buffer (8 ml) and incubated at room temperature for 6 h (with gentle rocking).

3. High titre stocks were harvested from the plates using a 1 ml pipette and transferred to 15 ml glass COREX tubes. After a 5 min spin at 9500 rpm the resulting supernatants were dispensed into 1.5 ml screwcap tubes and stored at 4°C.

4. For long term storage, 100 µl aliquots of each high titre stock were mixed with DMSO (at a final concentration of 8%), flash frozen in liquid N_2 and transferred to -70°C.

2.2 ISOLATION OF BACTERIOPHAGE DNA

2.2.1 Method

1. High titre stock from each putative periaxin gene clone (1 ml) was transferred to a large microfuge tube containing 350 µl of 3M NaCl/30% PEG, mixed by inversion and incubated on ice for 30 min.

2. Following a 10 min centrifugation at 13000 rpm and 4°C, the resulting phage pellets were resuspended in 250 µl SM buffer. DNase I (2.5 µl of a 1 mg/ml stock; SIGMA) was then added and the tubes incubated at 37°C for 30 min (with occasional vortexing).

4. An equal volume of lysis solution (0.3 M Tris HCl pH 9, 100 mM EDTA, 1.25% SDS) was subsequently dispensed into each tube, mixed by inversion, and incubated at 65°C for 10 min (with occasional mixing).

5. Neutralizing solution (250 µl; 3M potassium acetate pH 5.5) was then added, mixed thoroughly by inversion, and the tubes incubated on ice for 10 min.

6. After a 5 min centrifugation at 13000 rpm and 4°C supernatants were transferred to fresh microfuge tubes and extracted twice with phenol-chloroform (1:1) and once with chloroform.

7. Isopropyl alcohol (0.8 vol) was then added and the tubes incubated on ice for 30 min before centrifugation at 13000 rpm and 4°C for 10 min.

8. The phage DNA pellets were subsequently washed in 70% ethanol then allowed to dry. The DNA was then gently suspended in 20 µl TE/RNase (10 mM Tris HCl pH 8.0, 1 mM EDTA, 2% RNase cocktail (Ambion)).

9. Tubes were left at room temperature overnight to allow the DNA to dissolve completely.

2.3 EXCISION AND SUBCLONING OF GENOMIC DNA INSERTS FROM BACTERIOPHAGE LAMBDA DASH II

2.3.1 Method

1. DNA from each putative positive clone was digested with *Eco*R1.

In a 0.5 ml microfuge tube:-

3.0 µl λDNA

1.5 µl Buffer H

9.5 µl Sterile milli-Q water

0.5 µl*Eco*R1 restriction enzyme (10 U; New England Biolabs)

The tube was then incubated overnight at 37°C.

2. Digests were subsequently electrophoresed on 0.8% agarose gels in TAE containing 0.5 µg/ml EtBr and visualized by UV transillumination.

3. A comparison of the sizes of the resulting *Eco*R1 fragments in all of the clones demonstrated 3 distinct clone types. The *Eco*R1 fragments from a representative clone of each type were subsequently excised from the gel and purified by Qiaex II (Qiagen) according to manufacturers instructions.

4. The following ligation was set up for each purified *Eco*R1 fragment:-

Purified fragment DNA (vacuum-dried)

1.0 µl *Eco*R1-dephosphorylated pIBI30 (Qiagen)

0.5 µl 10x ligase buffer (Promega)

3.0 µl Sterile milli-Q water

0.5 µl T4 DNA ligase (0.5 U; Promega)

The reaction was then incubated overnight at room temperature.

4. Ligations were subsequently transformed into $CaCl_2$-competent XL1-blue (100 µl) and plated onto LB agar (1.5%; Gibco BRL) containing ampicillin (0.1 mg/ml), X-gal (0.008%) and IPTG (300 µM).

5. Resulting white colonies were inoculated into LB-broth (5 ml) containing ampicillin (0.1 mg/ml) and incubated overnight at 37°C with shaking.

6. Cultures were spun for 30 sec at 13000 rpm and the supernatants discarded. The bacterial pellets were then resuspended in 300 µl TELT (50 mM Tris HCl pH 8, 4% Triton X-100, 62.5 mM EDTA, 2.5 M LiCl) by vortexing for 15 sec. An equal volume of phenol-chloroform was then added, mixed by vortexing for 10 sec and spun for 3 min at 13000 rpm.

7. Supernatants (270 µl) were transferred to fresh tubes and 3 vols of 0.1 M sodium acetate in absolute ethanol added. After a brief mix, the tubes were spun for 45 min at room temperature.

8. The resulting pellets were washed in 70% ethanol, allowed to dry and then resuspended in 20 µl TE. The presence of a correct insert was subsequently determined by restriction digest with *Eco*R1 followed by electrophoresis on an agarose in TAE gel.

2.4 SEQUENCING OF GENOMIC SUBCLONES

2.4.1 Special reagents and materials

Sequencing primers

Short (15-25 nt) oligonucleotides for sequencing and PCR were synthesized on a model 391 PCR-MATE (Applied Biosystems) and purified by butanol extraction (Sawadogo and VanDyke, 1991)

2.4.2 Method

Template Denaturation

1. In a 0.5 ml microfuge tube:-

   3 µg Plasmid DNA

   3 µl NaOH (2 M)

   Sterile milli-Q water to 15 µl.

2. After 20 min at room temperature, 1M sodium acetate pH 4.8 (15 µl) and absolute ethanol (90 µl) was added and the tube incubated at -70°C for 30 min.

3. Following a 15 min spin at 13000 rpm (4°C), the DNA pellet was washed in 70% ethanol and vacuum dried.

Primer annealing

DNA pellets were resuspended in milli-Q water (5 µl) and the following additions made:-

1 µl Sequencing primer (5 µM)

1 µl Annealing buffer

The tube was then incubated at 37°C for 20 min and at room temperature for 10 min.

Labelling reactions

The following were added to the tube containing the annealed template and primer:-

1.5 µl Labelling mix A

0.3 µl Sterile milli-Q water

0.2 µl [α-^{35}S]dATP (ICN Pharmaceuticals)

1.0 µl Diluted T7 DNA polymerase (1:4 with dilution buffer)

The tube was then incubated at room temperature for 4 min.

Termination reactions

1. After incubation, 4.5 µl was transferred to each prewarmed (39°C) sequencing mix (1.3 µl). The tubes were then incubated at 39°C for 5 min.

2. Stop solution (2.5 µl) was then added and the tubes immediately frozen at -20°C.

Note. All reagents used were from a T7 sequencing kit (Pharmacia) unless otherwise stated.

Gel electrophoresis

1. Reactions were boiled for 3 min then immediately loaded (2.5 µl) on adjacent lanes of a sequencing gel (6% (v/v) acrylamide/bisacrylamide (19:1), 42% (w/v) urea, 1 xTBE (0.09 M Tris HCl, 0.09 M boric acid, 2 mM EDTA pH 8.0), TEMED (0.001% (v/v)), ammonium persulphate (0.02% (w/v)) assembled in an IBI Base Runner Nucleic Acid Sequencer (60 cm x 21.6 cm plates) according to the manufacturers instructions and with buffer reservoirs filled with 1x TBE. Electrophoresis was for approximately 4 h at 60 W.

5. The sequencing gel was fixed for 15 min in methanol (5%) and acetic acid (5%), blotted on 3MM paper and dried under vacuum for 90 min at 80°C.

6. The dried gel was then exposed to X-ray film (Fuji RX) for 48 h at room temperature.

7. The autoradiograph was developed using LX24 X-ray developer and Industrex manual fixer (both Kodak).

8. Sequences were analyzed using Macvector sequence analysis software (Oxford Molecular Group) and the University of Wisconsin GCG package (Devereaux et al, 1984).

2.5 SOUTHERN BLOT ANALYSIS

2.5.1 Method

1. DNA samples were electrophoresed on a 1% agarose gel in TAE for approximately 4 h at 55V.

2. Resolved DNAs were subsequently transferred to Magna nylon membrane (Micron Separations Inc.) using a vacuum blotting unit (VacuGene XL, Pharmacia Biotech), according to manufacturers instructions. Transfer included 5 min in depurination solution (0.25 M HCl), 10 min in denaturing solution (1.5 M NaCl, 0.5 M NaOH), 10 min in neutralization solution (1.5 M NaCl, 0.5 M Tris HCl pH 8.0) and 2 h in 20x SSC. The membrane was then baked at 80°C for 2 h.

4. After briefly wetting with distilled water, the membrane was arranged in a 50 ml polypropylene tube, covered in 3.5 ml of Rapid-Hyb buffer (Amersham) and incubated at 65°C for 30 min (with constant mixing). Labelled probe was then added to the tube and the incubation continued for a further 1.5 h.

5. The membrane was subsequently washed twice in 2x SSC/0.1% SDS (10 min per wash; room temperature) and twice in 0.2x SSC/0.1% SDS (30 min per wash; 65°C).

6. After sealing in food wrap (SARAN), the blot was exposed to X-ray film (Fuji RX) for 1 week at room temperature.

7. The autoradiograph was developed using LX24 X-ray developer and Industrex manual fixer (both Kodak).

2.6 5'-RACE (Rapid Amplification of cDNA Ends)

2.6.1 Special reagents and materials

Sciatic nerve total RNA.

Total RNA was extracted from the sciatic nerves of 15-day-old mice using RNAzol B (AMS Biotechnology) according to the manufacturers instructions. An aliquot (2 µl) of the RNA solution was subsequently diluted to 1 ml with milli-Q water and the absorbance at 260 nm measured. The concentration of RNA was then calculated (1 OD = 40 µg/ml RNA).

2.6.2 Method

Reverse Transcription

1. The following were dispensed into a 0.5 ml microfuge tube:-

   1 µl Random primers (150 ng; Gibco BRL)

   5 µg Total RNA

   Sterile DEPC-treated milli-Q water to 12 µl

The tube was then incubated at 70°C for 10 min then immediately quenched on ice.

2. After a brief centrifugation the following additions were made:-

   4 µl 5x first strand synthesis buffer (Gibco BRL)

   1 µl dNTP mix (each 10 mM)

   2 µl DTT (100 mM)

The tube was subsequently incubated at 42°C for 2 min.

3. Superscript reverse transcriptase (200 U; Gibco BRL) was then added and the reaction incubated at 42°C for 45 min.

4. Following single phenol-chloroform and chloroform extractions, the cDNA was precipitated by the addition of 3 vols of 0.1 M sodium acetate in absolute ethanol and then pelletted by centrifugation at 13000 rpm for 45 min at 4°C.

5. After a wash in 70% ethanol, the pellet was vacuum-dried and then resuspended in 10 µl DEPC-treated milli-Q water.

Terminal Deoxynucleotidyl Transferase (TdT) tailing of cDNA

1. cDNA (10 µl) was incubated at 70°C for 10 min then immediately quenched on ice.

2. After a brief centrifugation, the following additions were made:-

2 µl 5x first strand synthesis buffer (Gibco BRL)

7 µl dCTP (0.5 mM)

0.8 µl TdT (10 U; Gibco BRL)

The reaction was then incubated at 37°C for exactly 10 min.

3. The TdT was subsequently heat inactivated for 10 min at 70°C and the tailed cDNA then kept on ice.

PCR amplification of poly(C) tailed cDNA

1. In a 0.5 ml microfuge tube:-

1.0 µl Tailed cDNA

2.5 µl LMD30 primer (5 µM; nts 426-406 of rat periaxin - see appendix)

2.5 µl 5' RACE anchor primer (5 µM; see appendix)

2.5 µl 10x reaction buffer

2.5 µl dNTP mix (each 2 mM)

13 µl sterile milli-Q water

1.0 µl Dynazyme polymerase (Flowgen; diluted to 0.25 U/µl with 1x reaction buffer)

The reactions were then overlaid with mineral oil (15 µl) and subjected to the following cycle conditions:-

94°C for 1 min; 60°C for 1 min; 72°C for 1 min } first 5 cycles

94°C for 40 sec; 60°C for 1 min; 72°C for 1 min } next 30 cycles

94°C for 40 sec; 60°C for 1 min; 72°C for 4 min } last cycle

2. After chloroform extraction, PCR products were electrophoresed on a 1% agarose gel in TAE. Major products were excised, purified by Qiaex II and subcloned into the pGEM T-Vector (Promega) according to manufacturers instructions.

3. Subcloned PCR products were subsequently sequenced as before (see section 2.4).

2.7 SCREENING OF cDNA LIBRARY

2.7.1 Special reagents and materials

cDNA Library.

The cDNA library screened during this procedure was the same as that used by Gillespie et al (1994) for the isolation of rat periaxin 5' sequence; sciatic nerve poly(A)$^+$ RNA from 15-day-old rats was reverse transcribed with random hexamers and the resulting cDNA cloned into the λgt11 vector (Promega; see appendix) before packaging with Gigapack Gold packaging extracts (Stratagene).

NB. Before use, it was necessary to determine a current titre for the library, following its extended storage at 4°C.

Host Bacterium.

NZY broth containing maltose (0.2%) and ampicillin (0.1 mg/ml; Boehringer Mannheim) was inoculated with bacterial strain Y1090 (Promega) then incubated at 37°C for 6 h with shaking.

2.7.2 Method

Clones (3×10^5) from the above library were screened by plaque hybridization essentially as described in 2.1 except with bacterial strain Y1090 as the host and using a restriction fragment spanning nts 1-599 of the 4.6 kb rat periaxin pA1BG clone (Gillespie et al 1994) as the probe. The hybridization temperature was also 65°C.

2.8 PRIMER EXTENSION

2.8.1 Method

Primer labelling

The following were dispensed into a 0.5 ml microfuge tube:-

1 µl CSG7 primer (10 µM; nts 547-569 of rat periaxin - see appendix)

2 µl 5x T4 polynucleotide kinase reaction buffer (Gibco BRL)

2.5 µl [γ-^{32}P]ATP (ICN Pharmaceuticals)

4 µl Sterile milli-Q water

0.5 µl T4 polynucleotide kinase (5 U; Gibco BRL)

The tube was then incubated at 37°C for 45 min and 65°C for 10 min.

Hybridization

1. The following were dispensed into a 0.5 ml microfuge tube:-

10 µg Mouse trigeminal nerve total RNA

5 µl Labelled primer (from above)

Sterile milli-Q water to 10 µl

1 µl Sodium acetate (3 M)

28 µl ethanol (100%)

The tube was then incubated at -70°C for 30 min.

2. Following a 30 min spin at 13000 rpm and 4°C, the pellet was washed in 70% ethanol and respun (2 min).

3. After drying, the pellet was resuspended in 10 µl formamide hybridization buffer (0.9% formamide in 2.6x PIPES). The tube was then heated at 80°C for 10 min before overnight incubation at 42°C.

4. Sterile milli-Q water (57 µl) and 100% ethanol (135 µl) was subsequently added and the tube incubated at -70°C for 30 min.

5. Following a 30 min spin at 13000 rpm and 4°C, the pellet was washed in 70% ethanol and respun (2 min). The pellet was then allowed to dry.

Reverse transcription

1. The pellet from the above step was resuspended in the following:-

   7 µl Sterile milli-Q water

   4 µl 5x first strand synthesis buffer (Gibco BRL)

   2 µl DTT (0.1 M)

   5 µl dNTP mix (each 2 mM)

   1 µl Actinomycin D (1 µg/µl)

Superscript reverse transcriptase (200 U; Gibco BRL) was then added and the reaction incubated at 42°C for 45 min.

2. Following ethanol precipitation as before, the resulting pellet was dissolved in TE (10 mM Tris HCl pH 8.0, 1 mM EDTA; 3 µl) and Stop solution (T7 sequencing kit, Pharmacia; 3µl).

3. An aliquot of this solution (2.5 µl) was subsequently electrophoresed on a 6% sequencing gel (see 2.4.2) against a sequencing ladder comprising the 5'-end of mouse periaxin (generated by priming the longest mouse periaxin subclone from 2.6 with CSG20 (nts 103-122 of mouse periaxin - see appendix)

2.9 NORTHERN BLOT ANALYSIS

2.9.1 Method

1. Vacuum-dried RNA was resuspended in sample buffer (10 µl; 1x MOPS [0.02 M MOPS, 5 mM sodium acetate, 10 mM EDTA pH 7], 50% formamide, 7.4% formaldehyde, 0.05 µg/µl ethidium bromide), incubated for 15 min at 65°C and then quenched on ice.

2. After the addition of sterile loading buffer (1 µl) samples were electrophoresed on a 0.8% agarose gel containing 1x MOPS and formaldehyde (1.1%) in a 1x MOPS running buffer (Kroczek & Siebert 1990) for approximately 4 h at 55V.

3. Separated RNAs were subsequently vacuum-transferred to Magna nylon membrane (Micron Separations Inc.) for 2 h in 20x SSC using a vacuum blotting unit (VacuGene XL, Pharmacia Biotech), according to the manufacturers instructions. The membrane was then baked at 80°C for 2 h.

4. After briefly wetting with distilled water, the membrane was arranged in a 50 ml polypropylene tube, covered in 3.5 ml of Rapid-Hyb buffer (Amersham) and incubated at 65°C for 30 min (with constant mixing). Labelled probe was then added to the tube and the incubation continued for a further 1.5 h.

5. The membrane was subsequently washed twice in 2x SSC/0.1% SDS (10 min per wash; room temperature) and twice in 0.2x SSC/0.1% SDS (30 min per wash; 65°C).

6. After sealing in food wrap (SARAN), the blot was exposed to X-ray film (Fuji RX) for 1 week at room temperature.

7. The autoradiograph was developed using LX24 X-ray developer and Industrex manual fixer (both Kodak).

2.10 SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

2.10.1 Special reagents and materials

Sciatic nerve protein.

Sciatic nerves were taken from 15-day-old mice and solubilized by boiling in a minimum volume of 2% (w/v) SDS containing proteinase inhibitors (10 µg/ml leupeptin and 10 µg/ml antipain (both SIGMA)) for 3 min. Following a 5 min centrifugation at 13000 rpm and 4°C, the resulting supernatant was dispensed into small aliquots, fresh proteinase inhibitors added and flash frozen before storage at -40°C. An aliquot from each preparation was subsequently taken and its protein content estimated using a modified Lowry (Lowry et al 1951):-

Protein standards of 0-15 µg Bovine Serum Albumin (BSA; New England Biolabs) in 2% (w/v) SDS with proteinase inhibitors (100 µl) and dilutions of the nerve preparation (in 100 µl of the same solution) were prepared in duplicate in 2 ml microfuge tubes. Following the addition of Reagent A (125 µl; 1M NaOH, 0.25% SDS) the reactions were incubated at 60°C for 15 min. Fresh Reagent B (1.25 ml; 0.02% (w/v) potassium sodium tartrate, 0.01% (w/v) $CuSO_4$, 2% (w/v) Na_2CO_3) was then added and each tube incubated at room temperature for a further 15 min. Finally, 125 µl of a fresh 1N solution of Folin-Ciocalteu phenol reagent (Fisons) was added and mixed rapidly by inversion. After a 45 min incubation at room temperature the A_{750} of each reaction was measured. A standard curve was subsequently generated, from which the protein content of the nerve preparation was estimated.

4-20% linear gradient polyacrylamide gels.

Linear gradient resolving gels (4-20%) were cast using an SE 615 multiple casting chamber (Hoefer Scientific Instruments) according to manufacturers instructions and based on the buffer system of (Laemmli 1970). The resulting gels were held between 18 x 16 cm glass plates and measured 14 cm x 12 cm x 1.5 mm thick.

For 10 gels:-

	4% Stock	20% Stock
30% (w/v) acrylamide/		
bis-Acrylamide (37.5:1)(Anachem)	16.7 ml	83.3 ml
Tris-HCl, pH 8.9 (1.5 M)	25.0 ml	25.0 ml
SDS (20%)	0.625 ml	0.625 ml
N,N,N',N',-Tetramethylethylene diamine (TEMED)	0.045 ml	0.045 ml
milli-Q water	83.3 ml	16.7 ml
Ammonium persulphate (10% (w/v); fresh)	0.3 ml	0.06 ml
Final volume	125 ml	125 ml

A stacking gel was added to each individual resolving gel immediately prior to electrophoresis. Per stacking gel:-

2.25 ml acrylamide/bis-acrylamide (37.5:1; 30%)

1.875 ml Tris HCl pH 6.7 (0.5 M)

75 µl SDS (20%)

7.5 µl TEMED

Sterile milli-Q water to 15 ml

150 µl fresh ammonium persulphate (10%)

Immediately following the addition of the ammonium persulfate this solution was rapidly pipetted into the space above the resolving gel to a level approximately 2 cm from the top.

2.10.2 Method

1. Sciatic nerve protein from 15-day-old mouse (100 µg) and PNS myelin from 16-day-old rat (20 µg; A gift from Dr S. Gillespie) were dispensed into separate screwcap tubes and the following additions made:-

330 µl 3x Sample buffer (1% bromophenol blue, 20% glycerol, 0.2 M Tris-HCl , 8% SDS)

167 µl dithiothreitol (DTT; SIGMA; 1M)

100 µl 10x proteinase inhibitors (0.1 mg/ml leupeptin and 0.1 mg/ml antipain)

Sterile milli-Q water to 1 ml

2. After boiling for 2 min the samples were spun for 5 min at 13000 rpm and 4°C.

3. The resulting supernatants were loaded across the surfaces of separate 4-20% linear gradient polyacrylamide gels (see above) assembled in a SE 400 Sturdier Slab Gel Electrophoresis Unit (Hoefer Scientific Instruments) according to the manufacturers instructions and with buffer reservoirs filled with a solution containing Tris-HCl pH 8.4 (12.5 mM), glycine (96 mM) and 0.1% SDS.

4. Proteins were subsequently electrophoresed for approximately 4 h at a current of 30 mA (until the bromophenol blue dye in the sample buffer was just off the bottom of the gel).

2.11 WESTERN BLOT ANALYSIS

2.11.1 Method

1. Following SDS-PAGE, separated proteins were electrophoretically transferred to 0.45 µm nitrocellulose (Schleicher and Schuell) for 2 h at 45 mA in a buffer containing Tris-HCl pH 8.4 (25 mM), glycine (192 mM) and 20% (v/v) methanol, using a TE22 Mighty Small Transphor Electrophoresis Unit (Hoefer Scientific Instruments) according to the manufacturers instructions (Towbin et al 1979).

2. When the transfer was complete the nitrocellulose membrane was rinsed thoroughly in 1x PBS then blocked for 3 h in a blocking buffer of 1x PBS containing 0.2% (w/v) gelatin and 0.1% (v/v) Triton X-100.

3. Thin (3-5 mm) strips were subsequently cut from the filter and incubated for 1.5 h at room temperature in a solution of the appropriate primary antibody diluted in blocking buffer.

4. Following 3 x 5 min washes in blocking buffer, the strips were incubated with a solution containing a horse radish peroxidase (HRP)-conjugated donkey anti-rabbit IgG secondary antibody (Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke), diluted 1:500 in blocking buffer.

5. After 1 h at room temperature the strips were washed once in blocking buffer and then twice in 1x PBS.

6. Immunoreactive proteins were visualized by incubating in a solution of 50 mM Tris-HCl pH7.4 containing 1 mg/ml of 3,3'- diaminobenzidine (DAB; SIGMA) and adding 30% (v/v) H_2O_2 (1 µl).

7. When sufficiently developed, the peroxidase reaction was stopped by the addition of SDS to 2% (v/v).

2.12 IN VITRO TRANSCRIPTION

2.12.1 Special reagents and materials

Linearized template DNAs.

Linearized template DNA for the *in vitro* transcription of the 4.6 kb periaxin mRNA was generated from the pA1BG plasmid (Gillespie et al 1994). For the transcription of the 5.2 kb periaxin mRNA, template DNA was generated from a modified pA1BG plasmid in which the 0.2 kb *Sst*II-*Hinc*II restriction fragment between nts 633 and 734 of the 4.6 kb periaxin cDNA had been replaced with a 0.8

kb *Sst*II-*Hinc*II fragment from the partial 5.2 kb periaxin cDNA clone F4 (which includes the entire intron 6 sequence)

To prepare the linearized template DNAs, the following were dispensed into a 0.5 ml microfuge tube:-

10 µg plasmid midiprep DNA

5 µl Buffer H (Boehringer Mannheim)

Sterile milli-Q water to 47 µl.

*Not*I restriction enzyme (30 U; New England Biolabs) was then added, mixed gently, and the tube incubated at 37°C for 6 h. After taking the volume to 200 µl with TE (10 mM Tris HCl pH 8.0, 1 mM EDTA), each digest was extracted once with phenol-chloroform and then once with chloroform before precipitation with 0.1 M sodium acetate in absolute ethanol. The resulting DNA was suspended in 10 µl of DEPC-treated milli-Q water. The concentration of linearized template was subsequently determined by a visual comparison with a known standard following electrophoresis on a 1% agarose gel in TAE.

2.12.2 Method

1. The following were dispensed into a 0.5 ml microfuge tube (on ice):-

1µg linearized template DNA (see above)

2 µl 10x transcription buffer (Boehringer Mannheim)

2 µl ATP (10mM)

2 µl CTP (10mM)

2 µl TTP (10mM)

2 µl GTP (10mM)

2 µl RNA CAP Structure Analog (7 mG(5')ppp(5')G; 5 mM; New England Biolabs)

1 µl RNasin RNase inhibitor (40 U; Promega)

DEPC-treated milli-Q water to 19 µl

T7 RNA polymerase (15 U; Promega) was then added and the reaction incubated for 1 h at 37°C.

2. RQ1 RNase-free DNase (2 U; Promega) was subsequently added and the tube returned to 37°C for a further 15 min.

3. After taking the volume to 200 µl with DEPC-treated milli-Q water, the transcriptions were extracted once with phenol-chloroform and then once with chloroform.

4. RNA was precipitated by the addition of one tenth vol of 3M sodium acetate pH 4.8 and 1 vol. of isopropylalcohol followed by a 1 h incubation on ice.

5. After a 45 min spin at 13000 rpm and 4°C, the resulting RNA pellet was gently washed (not resuspended) in 500 µl of ethanol (diluted to 70% with DEPC-treated milli-Q water) and spun for 5 min at 13000 rpm.

6. The RNA was then allowed to air dry before finally suspending in 20 µl of DEPC-treated milli-Q water. A fraction of this solution (4 µl) was subsequently diluted to 1 ml with milli-Q water and the absorbance at 260 nm measured. The concentration of RNA was then calculated (1 OD = 40 µg/ml RNA).

7. To confirm the integrity and relative quantities of the transcribed RNAs, a sample of each (1 µg) was electrophoresed on a 0.8% agarose formadehyde gel (see section 2.9).

2.13 IN VITRO TRANSLATION

2.13.1 Method

1. Translation of the *in vitro* transcribed RNAs from 2.12 was carried out using the 'Flexi' rabbit reticulocyte system (Promega) according to the manufacturer's instructions. Briefly:-

In vitro transcribed RNA (0.5 µg) corresponding to either the 4.6 or 5.2 kb periaxin mRNA was dispensed into a 0.5 ml microfuge tube, denatured at 67°C for 10 min

then quenched on ice. After a short pulse spin the following additions were made (on ice):-

0.5 µl Amino Acid Mixture Minus Methionine (1mM)

0.7 µl KCl (2.5 M)

0-2 µl Magnesium acetate (25 mM; see notes)

0.5 µl RNasin RNase inhibitor (40 U; Promega)

2.0 µl ^{35}S-methionine (22.74 µCi; ICN Radiochemicals)

DEPC-treated milli-Q water to 8.5 µl.

An aliquot of reticulocyte lysate was then thawed and 16.5 µl added rapidly to the tube. After a gentle mix, the reaction was incubated at 30°C for 1.5 h.

2. An aliquot (1 µl) of the completed translation was then spotted onto a small circle of Whatman filter and allowed to dry. The remainder was flash frozen in liquid N_2 and stored at -40°C.

3. After washing the filter in a large excess of ice-cold 10% (w/v) trichloroacetic acid (TCA) for 10 min and then in a large excess of ice-cold 5% (v/v) TCA for 5 min the filter was then transferred to boiling 5% (v/v) TCA and boiled for 15 min.

4. Following two further 5 min washes in ice-cold 5% (v/v) TCA and 2 x 3 min dehydrations in excess absolute ethanol, the filter was allowed to dry.

5. Hydrogen peroxide (5 µl of a 15% (v/v) solution) was then spotted onto the dry filter and left for 30 min. This resulted in a decolourization of the translation (so as to minimize colour quenching during scintillation counting).

6. After repeating the H_2O_2 bleaching, the filter was pushed to the bottom of a 1.5 ml microfuge tube and covered with 1 ml of liquid scintillant (Opti phase 'Hi-Safe', Wallac). Radioactivity was then measured on a scintillation counter.

2.13.2 Notes

- Titration of $Mg2^+$ between 0-5 mM was required to achieve optimal translations of the individual RNAs. It should be noted that the endogenous $Mg2^+$ level of the lysate displayed batch-to-batch variability and therefore re-titration was necessary whenever a new batch was used.
- Background ^{35}S-met incorporation was calculated from a translation reaction in which template RNA had been omitted.

2.14 IMMUNOPRECIPITATION

2.14.1 Special reagents and materials

Protein A agarose

Protein A agarose (25 mg; SIGMA) was swollen for 1 h in Solution A (250 µl; 50 mM Tris-HCl pH 7.4, 2.5% (v/v) Triton X-100, 150 mM NaCl, 4 mM EDTA) then centrifuged for 5 min at 13000 rpm. After discarding the supernatant, the pellet was resuspended in 1 ml of Solution A. The tube was then spun again to repellet. This wash step was repeated twice more. The final volume of the agarose was subsequently determined and 2 vol of Solution A added before storage at 4°C.

2.14.2 Method

1. The following were dispensed into a 1.5 ml screwcap microfuge tube:-

   50,000 cpm of 4.6 kb periaxin mRNA *in vitro* translation

   200,000 cpm of 5.2 kb periaxin mRNA *in vitro* translation

   200,000 cpm of a control *in vitro* translation (ELAV; a gift from Dr S. Tait)

   12.5 µl SDS (20%)

   2.5 µl 50x proteinase inhibitors (0.5 mg/ml leupeptin and 0.5 mg/ml antipain)

   Sterile milli-Q water to 125 µl

2. After boiling for 2.5 min, 4 vol. of Solution A containing 1x proteinase inhibitors was immediately added and mixed by inversion. The tube was then spun at 13000 rpm for 5 min.

3. The resulting supernatant was transferred to a fresh screwcap microfuge tube and protein A agarose (50 µl; see above) containing 1x proteinase inhibitors added. The tube was then rotated end-over-end on a bench-top windmill mixer for 1 h.

4. Following a 5 min spin the supernatant was again transferred to a fresh screwcap tube. Anti-NTerm antiserum (10 µl; rat periaxin amino acids 2-14 (Gillespie et al, 1994)) was then added and the tube rotated for a further hour.

5. After the addition of protein A agarose (50 µl; see above) the tube was returned to the windmill mixer, rotated for a third hour, and then spun for 5 min at 13000 rpm.

6. The resulting supernatant was then discarded and the pellet resuspended in 1 ml of Solution A containing 0.1% SDS. The tube was then spun for 5 min at 13000 rpm to repellet. This wash step was repeated twice more with Solution A containing SDS and then once with Solution A minus Triton X-100.

7. Following the final wash, the pellet was suspended in SDS-PAGE sample buffer containing 100 mM DTT (80 µl) then boiled for 3 min. After a 5 min spin at 13000 rpm, the supernatant was loaded onto a 4-20% gradient SDS-polyacrylamide gel (see section 2.10). Samples of the 4.6 kb mRNA *in vitro* translation (25,000 cpm), the 5.2 kb mRNA *in vitro* translation (100,000 cpm) and the control *in vitro* translation (100,000 cpm) were also loaded on the same gel. Electrophoresis was then carried out overnight at a current of 5 mA (See section 2.10).

8. When the bromophenol blue dye had reached the bottom of the gel, electrophoresis was stopped. At this point, the glass plates were separated, the gel removed and immersed for 30 min in Amplify (Amersham).

9. The gel was subsequently dried under vacuum for 2 h (with heat lamp) on a double thickness of 3MM paper and then exposed to X-ray film (Fuji RX) for 48 h at room temperature.

10. The autoradiograph was developed using LX24 X-ray developer and Industrex manual fixer (both Kodak).

2.14.3 Notes

- Only optimal translations were used (as defined by Mg2+ titration)
- All centrifugation and incubations were carried out at room temperature.
- All incubations during steps 2-5 were carried out in the presence of proteinase inhibitors (10 µg/ml leupeptin and 10 µg/ml antipain (both SIGMA)).

2.15 ANTIBODY PRODUCTION

2.15.1 Special reagents and materials

Dialysed keyhole limpet haemocyanin (KLH).

A length of seamless dialysis tubing (≥ 12,000 mol wt retention; SIGMA) was boiled for 5 min in approximately 500 ml of milli-Q water containing a small quantity (about a spatula tip-full) of $NaHCO_3$ and EDTA. When the water had cooled to room temperature, the tubing was rinsed thoroughly in an excess of fresh milli-Q water then one end was closed with a double knot. Using a Pasteur pipette, the tubing was then filled with 6 ml of a 10 mg/ml solution of KLH (produced by dissolving 60 mg of KLH powder (SIGMA) in 6 ml of sterile milli-Q water). After closing with a single knot, the tubing was suspended in 2.5 l of 1x PBS for 2 h at 4°C. The 1x PBS was then replaced with a fresh 2.5 l and the dialysis continued overnight. The following morning, the 1x PBS was replaced with 2.5 l of 10 mM potassium phosphate buffer (pH 7.0) and the dialysis continued for a further 6 h at 4°C. The tubing was then carefully re-opened with a cut just below the single knot

and the dialysed KLH transferred to a 15 ml glass COREX tube using a Pasteur pipette. Following a 10 min spin at 8500 rpm the supernatant was collected and the absorbance at 280 nm measured. The final concentration of KLH in the dialysed solution was then calculated ($E1\%^{280}$ = 18 OD). The KLH was then frozen and stored at -40°C until required.

Sephadex G25-50 gel-filtration column.

G25-50 Sephadex (12 g; SIGMA) was swollen for about 30 min in an excess of 0.1 M potassium phosphate buffer pH 6.0. The excess buffer was then poured off - down to the level of the swollen gel - and replaced with fresh. After swirling for a few seconds, the gel was allowed to sediment before the excess buffer was again poured off and replaced with fresh. This process was repeated twice more. Following the final wash, excess buffer was added, swirled quickly and the resulting gel slurry poured into a large glass column (4 cm in diameter x 19 cm high), secured in a vertical position with the tap at the bottom closed. When the gel had settled, the bottom tap was opened and 100-150 ml of buffer was gently applied to the column - this was then allowed to run through, down to a final level about 2-3 cm above the gel surface. The tap was then closed again and the column carefully moved to 4°C until required.

Sulpho-m-maleimidobenzoyl-N-hydroxysuccinimide (sulpho-MBS)-activated KLH.

Sulpho-MBS (12.5 mg; Pierce) was dissolved in 10 mM potassium phosphate buffer pH 7.0 (2.5 ml) then reacted with dialysed KLH (24 mg; see above) for 30 min at room temperature (with rotation). The G25-50 Sephadex column prepared above was taken from 4°C, the bottom tap opened, and the level of buffer allowed to drop to the surface of the gel. The sulpho-MBS-KLH was then carefully applied with a Pasteur pipette. After opening the bottom tap to allow this solution to just run into the gel, the tap was closed and the column filled with approximately 50 ml of 0.1 M potassium phosphate buffer pH 6.0, taking care not to disturb the surface of the gel. The tap was then re-opened and the eluate collected - the first 20 ml as a

bulk fraction which was discarded and the next 22.5-30 ml as 1.5 ml fractions (in 1.5 ml microfuge tubes) which were kept on ice. The absorbance of each fraction was subsequently measured at 280 nm - fractions were simply poured into the cuvette, the absorbance measured and the fraction then poured back into its microfuge tube (blank = 1st 1.5 ml fraction). A sharp peak in absorbance encompassing about 6-8 fractions was clearly visible, representing the elution of the MBS-activated KLH. These fractions were subsequently pooled and stored at -40°C until required.

2.15.2 Method

1. The peptide acyl-Ala-Lys-Leu-Val-Arg-Val-Leu-Ser-Pro-Val-Pro-Val-Gln-Asp-Ser-Pro-Ser-Asp-Arg-Val-Ala-Ala-Ala-Cys-amide (SPeri; 20 mg), synthesized by Prof. N. Groome, Department of Biology, Oxford Brookes University, and identical to amino acids 125 to 147 of the deduced mouse S-periaxin sequence (plus C-terminal cysteine residue for coupling), was dissolved in 10 mM HCl (2 ml) then extracted with ether (2 ml).

2. The lower aqueous phase from step 1 (containing the ether-extracted peptide) was subsequently transferred to a fresh tube and its pH adjusted to approximately 6.0 by the drop-wise addition of 0.1 M NaOH.

3. A sample (1.5 ml) of the peptide solution from the above step was then reacted with sulpho-MBS-activated KLH (2.5 ml; see above) at 4°C overnight (with rotation).

4. Ethanolamine (2 ml of 0.5 M) was then added to the KLH-coupled peptide from step 4 and the tube incubated for a further 1 h at 4°C. The solution was then dialysed twice against 1x PBS. (The dialysis procedure was essentially as described for the keyhole limpet haemocyanin up to the point of the dialysis against 10 mM potassium phosphate pH 7.0).

5. Dialysed KLH-coupled peptide (2.5 ml) was subsequently emulsified with Freund's complete adjuvant (3.3 ml; SIGMA) by vigorous shaking overnight at 4°C; the remainder was stored at -40°C until needed.

6. The resulting emulsion from the above step was used to immunize three New Zealand white rabbits (1.9 ml per rabbit: 0.5 ml injected intramuscularly into each hind limb; 0.9 ml injected subcutaneously into the scruff).

7. After 3 weeks, the immunization was repeated by injecting (in the same pattern as before) 1.9 ml of an emulsion prepared from the dialysed KLH-coupled peptide (2.5 ml) and Freund's incomplete adjuvant (3.3 ml; SIGMA).

8. Ten days later, a 2-5 ml test bleed was collected from each rabbit via an incision in the posterior marginal vein of the right ear.

9. After allowing the bloods to clot at room temperature for 1-2 h, the clot was detached from the sides of each container by ringing with a wooden cocktail stick. The bloods were then left overnight at 4°C to allow the serum to separate out.

10. The resulting serum were transferred to 15 ml COREX tubes using a Pasteur pipette and centrifuged at 8500 rpm for 15 min. The supernatants were then collected and stored as small aliquots at -40°C.

11. A 1:500 dilution of each of these test bleed antisera was subsequently used for western blotting protein from 15-day-old mouse sciatic nerves in order to determine the strength of immunoreactivity and specificity for S-periaxin in each animal (for a detailed method see section 2.11).

12. Specific but weak S-periaxin immunoreactivities as demonstrated by the above western blotting necessitated further reimmunization until a reasonably high antibody titre was achieved in at least one animal. To this end, all three animals were re-injected with a 1.9 ml of dialysed peptide-KLH and incomplete Freund's adjuvant emulsion one week after the initial test bleed. Steps 9-12 were then repeated.

13. Improved S-periaxin immunoreactivities demonstrated in step 13 led to a final immunization with a further 1.9 ml of dialysed peptide-KLH and incomplete Freund's adjuvant emulsion four days after the test bleeds from step 13.

14. Ten days later, rabbits were anaesthetized and fully bled by cardiac puncture using a 16G needle. The 80-100 ml of blood collected from each animal was then processed according to steps 9-11.

2.16 AFFINITY PURIFICATION

2.16.1 Special reagents and materials

Sepharose-SPeri peptide column .

A small, glass, gel filtration column (1.5 cm in diameter x 10 cm high) was secured in a vertical position with the bottom tap closed. Aminohexyl-sepharose 4B suspension (3 ml; SIGMA) was then added and allowed to settle. After opening the bottom tap, 1x PBS (10 ml) was pipetted into the column and allowed to run through. This wash was then repeated three more times and the tap closed. PBS (6 ml) and sulpho-MBS (500 µl; 5 mg/ml in dimethyl fluoride) were then added, the top sealed and the column incubated for 2 h at room temperature (with rotation). With the column secured back in an upright position, four further 10 ml washes with 1x PBS were carried out. After closing the bottom tap, PBS (2 ml) and SPeri peptide solution (2 ml; produced in essentially the same way as steps 1-3 of 'Antibody production' except that 15 mg of peptide was used instead of 20 mg) were then applied, and the column incubated at room temperature overnight (with rotation). In the morning, the column was secured upright, washed once with 1 M Tris-HCl pH 7.5 (1 ml), twice with 0.1M glycine pH 2.6 (10 ml) and twice with 1x PBS (10 ml). The column was then stored at 4°C until required.

2.16.2 Method

1. The Sepharose-SPeri peptide column (see above) was secured upright then washed once with 1 M Tris-HCl pH 7.5 (1 ml), twice with 0.1 M glycine pH 2.6 (10 ml) and twice with 1x PBS (10 ml).

2. PBS (1 ml) and final bleed antiserum (1 ml) from the rabbit giving the strongest immunoreactivity against S-periaxin (as determined by western blotting; see steps 11 and 14 of 'Antibody production') were then applied (with the bottom tap closed) and the column incubated for 2 h at room temperature (with rotation).

3. After securing upright, the column was washed through with an excess of 1x PBS. 0.1 M glycine pH 2.6 (15 ml) was then carefully applied to the column and the eluate collected as 1 ml fractions into large microfuge tubes containing 0.5 M Tris-HCl pH 7.5 (0.5 ml). Fraction 1 was subsequently discarded; the remaining 10 fractions were kept on ice until the level of S-periaxin immunoreactivity present in each was determined (by western blotting protein from 15-day-old mouse sciatic nerves with a 1:50 dilution - for a detailed method see section 2.11).

4. The 3 fractions from the above step which showed the highest S-periaxin immunoreactivity were pooled and stored as small aliquots at -40°C.

2.17 INDIRECT IMMUNOFLUORESCENCE

2.17.1 Special reagents and materials

3' Aminopropyl triethoxy-silane (TESPA)-subbed slides.

New, clean, glass microscope slides (BDH) were immersed in a 1N solution of hydrochloric acid in 70% ethanol for 20 sec, followed by a 20 sec wash in distilled water and a 20 sec wash in 100% acetone. After drying, slides were dipped in a 2% (v/v) TESPA solution (produced by diluting stock TESPA (SIGMA) to 2% with acetone) for 20 sec then washed twice in 100% acetone (each for 20 sec). When dry,

slides were stored (at room temperature) in a clean, sealed box containing desiccant until required.

2.17.2 Method

1. 20-day-old mice were anaesthetized then perfused by intracardiac injection of a 4% paraformaldehyde in 0.1 M sodium phosphate pH 7.4 (PFA) fixative. When completed, sciatic nerves were dissected out and fixed for a further 2 h at room temperature in an excess of PFA solution.

2. After 3 x 15 min washes in 0.1 M sodium phosphate buffer pH 7.4, nerves were cryoprotected by immersion for 15 min in a 5% (w/v) and then a 10% (w/v) solution of sucrose in phosphate buffer before incubating overnight in a 20% (w/v) sucrose solution at 4°C.

3. Cryoprotected nerves were then cut into 2-3 short lengths which were then frozen upright in OCT embedding compound (Tissue TEK) using liquid N_2-cooled isopentane.

4. Embedded nerves were subsequently cut into thin (7 µm) transverse sections and collected onto TESPA-subbed glass microscope slides (6 sections per slide). Slides were then allowed to dry for 1 h before further processing (or storage at -40°C).

5. Nerve sections were washed thoroughly with 1x PBS to remove residual OCT then blocked for 3 h at room temperature with a solution of 10% (v/v) goat serum (SAPU) in blocking buffer (1x PBS containing 0.2% gelatin and 0.1% Triton X-100).

6. Following blocking, sections were incubated overnight at room temperature with appropriate combinations of primary antibodies diluted in a solution of 4% goat serum in blocking buffer.

7. Following 4 x 5 min washes in blocking buffer, slides were incubated with secondary antibodies (FITC-conjugated goat anti-rabbit IgG (Cappel) at 1:200; biotinylated goat anti-mouse IgG at 1:500 (Kirkegaard and Perry Laboratories)),

diluted in 4% (v/v) goat serum in blocking buffer, for 1 h in the dark at room temperature. Slides were then washed three times in blocking buffer.

8. Slides were subsequently incubated in streptavidin-Texas red (Vector Laboratories), diluted to 1:1500 in blocking buffer containing 4% (v/v) goat serum, for 1 h in the dark at room temperature, then finally washed three times in 1x PBS.

9. A single drop of Vectashield mounting solution (Vector Laboratories) was then put on a coverslip and placed over the sections. Edges were then sealed with clear nail varnish.

10. Sections were subsequently examined with a Leica TCS4D confocal microscope.

2.17.3 Notes

- To minimize the quantities of precious antibodies and fluorochromes, incubation volumes during steps 5-8 were kept low (150-200 µl) by creating a small chamber over the tissue sections from a suitably sized coverslip raised at each end by a stack of two narrow glass strips (roughly 3 mm x 22 mm). The chamber was then filled gently by pipette. After each incubation the chamber was gently removed by flooding the slide with the relevant washing solution. All subsequent washes were carried out with the wash solutions in excess. A new chamber was therefore assembled at the beginning of each separate incubation.

- To prevent evaporation during incubations (especially overnight), slides were placed in a humid chamber (a large lidded petri dish with moistened 3MM paper on the bottom).

Results

3.1 STRUCTURE OF THE MURINE PERIAXIN GENE

3.1.1 Characterization of *Periaxin* Gene Clones

As described in detail in the Methods, a murine genomic library was screened with a 3 kb restriction fragment taken from a full-length rat periaxin cDNA (Gillespie et al, 1994). From 10^6 bacteriophage clones, 12 hybridization-positive clones were identified and isolated. A restriction digest carried out on phage DNA from each clone using *Eco*R1, revealed three independent clone types (Figure 13) - type 1 (F2, G1, G2, L1, N1 and T1) containing genomic inserts of approximately 15 kb, which gave rise to *Eco*R1 fragments of 11, 3.5, 0.6 and 0.1 kb, type 2 (B1, F4, I1, J1 and Q1) containing inserts of approximately 18.5 kb, which gave rise to *Eco*R1 fragments of 10.8, 5, 1.5 and 1.3 kb, and type 3 (H1) containing an insert of 22.5 kb, which gave rise to *Eco*R1 fragments of 11, 5.5, 3.5, 1.5 and 0.9 kb. The individual *Eco*R1 fragments from each of the three clone types were subcloned into pIBI30 and their ends sequenced (200-300 bp). By comparing these sequences, many of the fragments were shown to be identical to, or part of, fragments from the other clones demonstrating a large amount of overlap between the genomic inserts. Figure 14 presents the linear arrangement of the three clone types as deduced from these overlapping sequences, which cover a region of genomic DNA approximately 28 kb in length. It is important to note that the *Eco*R1 sites at the extreme ends of the clones are derived from the lambda multiple cloning region and therefore do not represent *Eco*R1 sites in the genomic DNA. Such sites were readily identifiable by the presence of an immediately adjacent *Sau*3A1 site (a characteristic feature of the library construction), which also allowed convenient orientation of the fragment.

Comparison of the sequences obtained from the ends of the *Eco*R1 fragments with the sequence of the periaxin cDNA demonstrated no similarity. This indicated that either the *periaxin* gene was located wholly within one of the isolated fragments or that the gene was split into a number of exons located within different fragments.

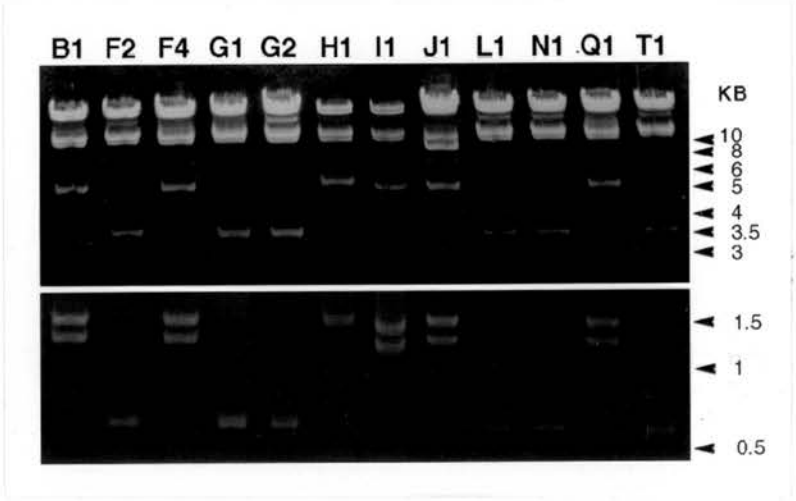


Figure 13. *Eco*R1 digestion of first screen genomic clones. Lambda DNA from each periaxin positive bacteriophage clone (12) was digested with *Eco*R1 and electrophoresed on both a 1.5% and 0.8% agarose gel in TAE. The small (<2 kb) *Eco*R1 fragments resolved on the 1.5% gel (bottom) and the large (>3.5 kb) fragments resolved on the 0.8% gel (top) indicate the presence of 3 independent clone types. The intense bands at 9 and 20 kb are the left and right arms of lambda.

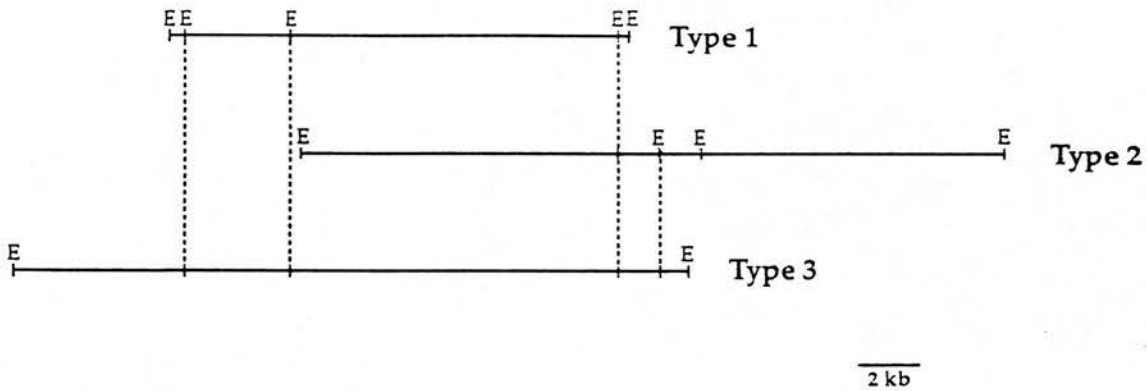


Figure 14. Schematic representation of the structural relationship of periaxin genomic clones. The 3 distinct periaxin-positive genomic clone types isolated from a Lambda DASH II phage library show a high degree of overlap as defined by *Eco*R1 digestion (E). Dotted lines indicate shared restriction fragments. Note: The EcoR1 sites shown at the ends of each clone are vector derived and therefore not present within the gene.

Southern blot hybridization of DNA from each distinct *Eco*R1 fragment with regional cDNA probes covering the entire rat periaxin sequence proved the latter to be the case, as 5' periaxin sequence was localized to the 5.5 kb *Eco*R1 fragment, and central and 3' sequence was localized to the 11 kb *Eco*R1 fragment (Figure 15). This finding also confirms the orientation, with respect to the periaxin cDNA, of the genomic DNA depicted in Figure 14.

The complete exonic-intronic organization of periaxin coding sequence within the 11 kb and and the 5.5 kb *Eco*R1 fragments was determined by PCR and sequencing, using periaxin sequence-specific oligonucleotide primers, with the rat 4.6 kb cDNA sequence as a reference. The mouse periaxin sequence identified within these fragments was shown to cover almost 94% of the gene from its homology to nts 300 to 4642 (the beginning of the poly(A) tail) of the published rat periaxin sequence. The absence of sequence homologous to nts 1-299 of the rat cDNA (which encode the 5' untranslated sequence together with the initiator methionine in rat periaxin) within the 5.5 kb fragment indicated two possibilities: either the corresponding mouse sequence was present within the 5.5 kb fragment but was just unrecognizable due to sequence divergence between the two species or, the homologous sequence was located on another library clone with sequence 5' to those isolated. Identification of the 5' end sequence of mouse periaxin by 5' RACE showed the first possibility to be untrue, with the 300 nts showing more than 94% homology to those of the rat. This meant the 5' end of the mouse *periaxin* gene was located on an as yet unidentified clone and hence necessitated a re-screen of the genomic library.

Using nts 52-300 of mouse periaxin as a probe (generated by PCR on the cDNA obtained by 5' RACE), a further 6×10^5 library bacteriophage were screened as before. From this, 2 hybridization-positive clones (BB1 and HH1) were obtained. An *Eco*R1 digest on DNA from each revealed a single independent clone type containing a 13.5 kb genomic insert, which gave rise to fragments of 7, 5 and 1.5 kb.

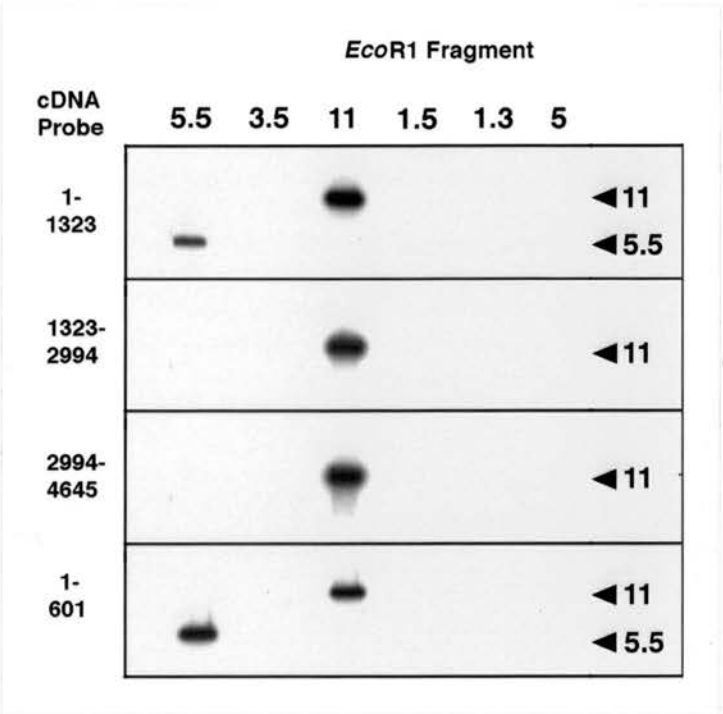


Figure 15. Southern blot hybridization of each distinct *Eco*R1 fragment from the type 1, 2 and 3 clones with regional cDNA probes covering the entire rat periaxin sequence. DNA from each subcloned fragment was digested with *Eco*R1, electrophoresed on 1% agarose in TAE gels, then transferred to nylon membranes. These membranes were subsequently probed with restriction fragments corresponding to nts 1-602, 1-1323, 1323-2994 and 2994-4645 of the published rat periaxin sequence, labelled with [α-^{32}P]dCTP by random priming. Sequence from the 5'-end of mouse periaxin is localized to the 5.5 kb *Eco*R1 fragment, whereas central and 3' sequences are localized to the 11 kb *Eco*R1 fragment. An intron of at least 3.5 kb separates these sequences.

Extensive characterization by PCR, sequencing and Southern blot hybridization successfully located the missing 300 nts of exonic sequence within these fragments, but also revealed no overlap between this clone and the clones from the previous screen. However, the HH1/BB1 and the H1 were shown to be contiguous by PCR performed on 129SV genomic DNA using a forward primer from the most 3' *Eco*R1 fragment of the HH1/BB1 clones (the 5 kb) and a reverse primer from the most 5' *Eco*R1 fragment of the H1 clone (the 5.5 kb). Therefore, in the gene, these 2 fragments (the 5 kb and the 5.5 kb) abut to form a region flanked by *Eco*R1 sites 10.5 kb apart (once again the *Eco*R1 sites at the end of the 5 kb and 5.5 kb clones are vector derived).

3.1.2 The Murine *Periaxin* Gene Contains 7 exons and Spans 20.6 kb

Figure 16 shows the complete structure of the murine *periaxin* gene (designated *Prx*) as deduced from all of the isolated clones. The gene spans a region of genomic DNA 20.6 kb in length - four and a half times the length of the 4.6 kb rat periaxin mRNA - and contains 7 exons interrupted by 6 introns. Exon size increases gradually through exons 1-6 (from 32 to 197 nts) which encode approximately 14% of the mRNA including the entire 5' untranslated (5' UTR) (exons 1-4), the initiation codon (exon 4) and the first 126 amino acids of the mouse periaxin protein (exons 4-6). Exon 7 is extremely large (4002 bp) and encompasses the remaining 86% of the gene up to and including the translation stop codon and the entire 3' untranslated region (3' UTR). The introns show similar extremes of size ranging from 88 bp (intron 2) to 7500 bp (intron 5). The nucleotide sequence around each exon-intron junction conforms to the canonical GT/AG rule for eukaryotic nuclear genes (Breathnach et al, 1978). This information is summarized in Table 2.

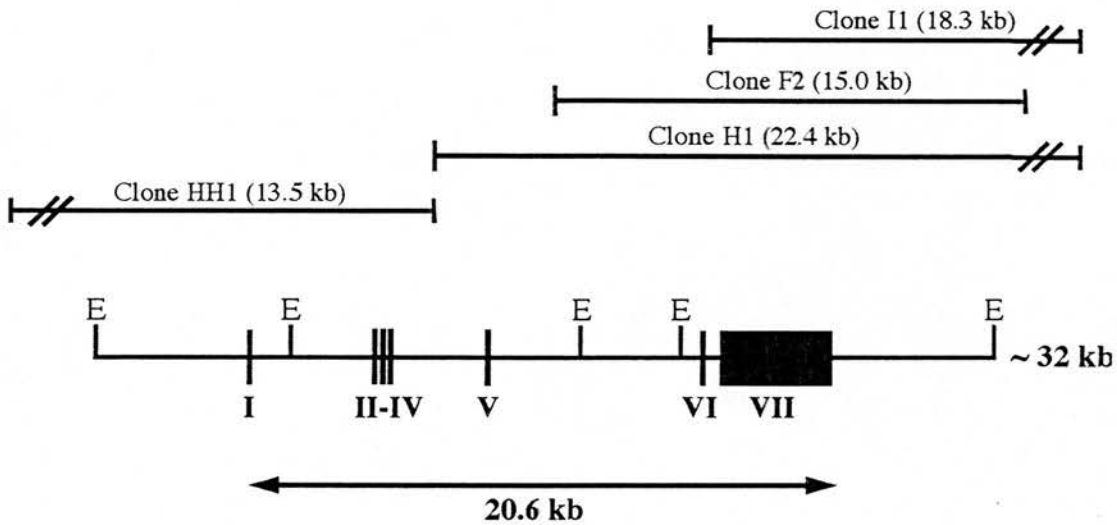


Figure 16. Structure of the murine periaxin gene. Exons are numbered and indicated by solid rectangles. Introns are shown as solid lines between the exons. *Eco*R1 (E) restriction sites are indicated and were used to subclone the genomic clones F2, H1 and I1 (from the first screen) and HH1 (from the second screen).

TABLE 2

Exon No. (size in bp)	cDNA position	5' Splice donor	Intron No. (size in bp)	3' Splice acceptor	Codon phase	Amino acid at splice site
1 (32)	1-32	CCACCG**gt**aaga	1(4900)	tcct**ag**AGCCCC	-	-
2 (44)	33-76	CCTCAG**gt**gaga	2(88)	tggc**ag**GCAGCA	-	-
3 (99)	77-175	GAAGCG**gt**gagg	3(116)	cccc**ag**GCTCAG	-	-
4 (126)	176-301	GCTGAG**gt**gggt	4(2900)	tcgc**ag**GAGCTG	0	9-Glu/Glu
5 (157)	302-458	AAGAAG**gt**aggc	5(7500)	ttgc**ag**GGGACC	1	62-Gly
6 (197)	459-655	AAGCTG**gt**acgc	6(592)	cctc**ag**AACATC	0	127-Leu/Asn
7 (4002)	656-4657					

Exon/intron position, size, and junction sequence structure of the mouse periaxin gene.

The intronic 5'-splice donor GT and the 3'-splice acceptor AG are bolded. The exonic sequences are capitalized. The interruption of codons by introns is indicated by the phase. A phase of 0 indicates no interruption, insertion of an intron after the first nucleotide of the codon is indicated by phase 1. All numbering is relative to the deduced cDNA sequence of the mouse 4.6 kb periaxin message.

3.1.3 *Prx* is a Single Copy Gene

When compared to the size range of introns and exons in an average vertebrate nuclear gene (Hawkins, 1988), the *Prx* intron sizes and the sizes of exons 1-6, are all typical. However, exon 7 is unusually long. To exclude the possibility that the genomic clones isolated encoded a mouse *periaxin* pseudogene, a genomic Southern blot was performed to characterize the *periaxin* gene in mouse chromosomal DNA. Figure 17 illustrates a Southern blot of 129SV genomic DNA cut with three different restriction enzymes and hybridized with a radiolabelled restriction fragment corresponding to nts 1323-4641 of the rat periaxin cDNA. Identified, are a single *Bam*H1 fragment, a single 11 kb *Eco*R1 fragment and a single 9.1 kb *Kpn*1 fragment. The number and size of each of these hybridizing fragments are precisely predicted from restriction maps of the type 1, 2 and 3 clones. This data, together with the recent mapping of the *periaxin* gene to a single locus on mouse chromosome 7 (Gillespie et al, 1997), would seem to indicate that the isolated clones do encode a bona fide, single copy mouse *periaxin* gene and that the final exon is actually very large.

3.2 SEQUENCE OF THE MOUSE PERIAXIN GENE

3.2.1 Sequence Comparison of rat and Mouse Periaxin

The sequences of all exons shows a very high degree of similarity to the sequence of the 4.6 kb rat periaxin cDNA used as a reference throughout the gene characterization (Gillespie et al, 1994; see appendix for full comparison). Overall, the mouse and rat periaxin DNA sequences are 93% identical. However, as can be seen from Figure 18, this belies a high degree of variability, with the similarity approaching almost 99% in some regions; elsewhere it is below 88%. This translates into an equally high degree of regional variability between the deduced amino acid

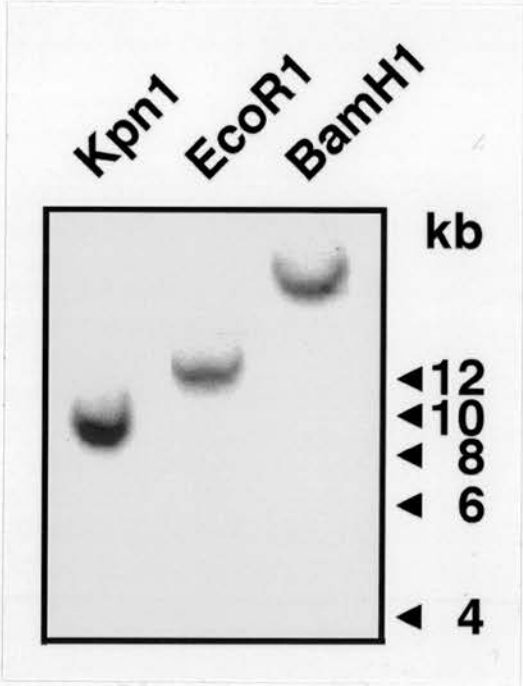


Figure 17. Periaxin is encoded by a single copy gene in the mouse. 129SV genomic DNA (10 μg) was digested with either EcoR1, BamH1 or Kpn1, electrophoresed on a 0.8% agarose in TAE gel, then transferred to nylon membrane. The membrane was subsequently probed with a restriction fragment corresponding to nts 1323-4641 of the rat periaxin cDNA. The number and size of each of these hybridizing fragments are precisely predicted from the restriction map of the type 1, 2 and 3 clones, indicating a single copy gene.

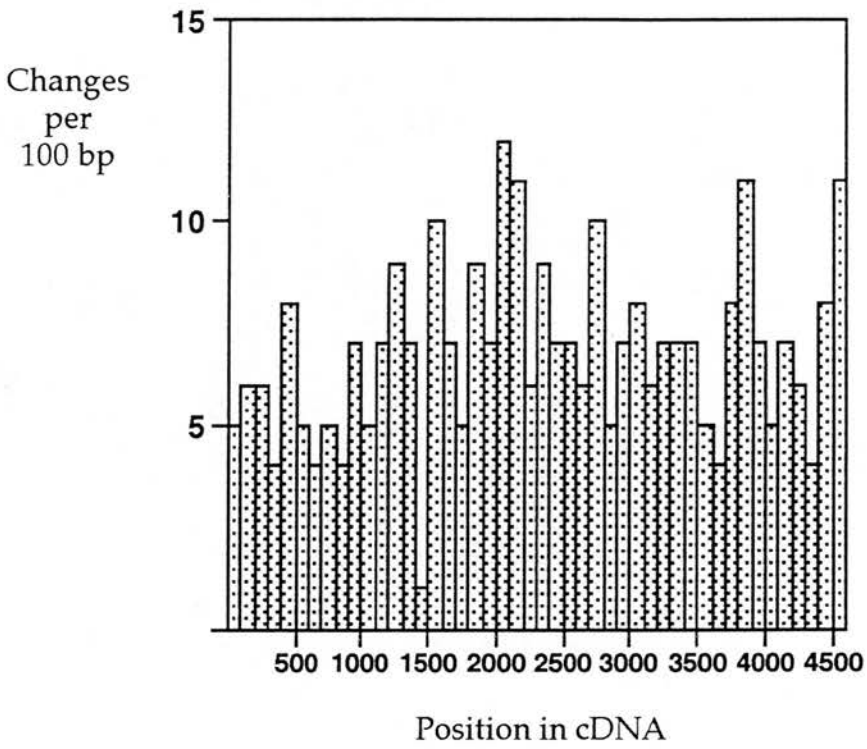


Figure 18. Quantitative analysis of the sequence similarity of the murine periaxin gene and the 4.6 kb rat periaxin cDNA. The number of nucleotide differences between rat and mouse periaxin was calculated at intervals of 100 bp. These values are plotted graphically as vertical bars. Overall, the sequences are 93% identical. However, regional variation is clearly visible.

sequence of the mouse periaxin (encoded by an mRNA comprising exons 1-7) and the deduced amino acid sequence of rat periaxin (Gillespie et al, 1994) (Figure 19A and B). The regions of highest and lowest similarity correspond well with the domain structure of rat periaxin as deduced from the primary sequence (Gillespie et al, 1994) (Figure 12). Conservation of the N-terminus (including the basic domain) and the acidic domain towards the C-terminus, would suggest that these regions are important to periaxin function.

The repeat domain (characterized by a repeating motif of a pentapeptide [aliphatic nonpolar:pro:glu or asp: aliphatic nonpolar:variable] and a tripeptide spacer (Gillespie et al, 1994) appears to show quite high variability. However, many of the changes result in the inclusion of a conserved amino acid. Given that this domain is thought to be physically inflexible (Gillespie et al, 1994) and has been postulated to play an important structural role in periaxin (extending the polypeptide and keeping the basic and acidic domains well separated), the inclusion of conserved amino acids presumably serves to maintain this structural property. Interestingly, the two species show some degree of polymorphism with respect to the repeating units. The repeat region of the mouse periaxin is larger than that of the rat by exactly two repeat units (which is necessary for retaining the correct open reading frame). This finding is most likely due to 'slipped-mispairing' during DNA replication (For review see Moore et al, 1983) and perhaps indicates the mechanism by which the periaxin repeat region has evolved to its present length, from a small number of ancestral units.

3.2.2 A Correction to the Published Rat Periaxin Sequence

A 19 bp sequence close to the 3' end of the coding region of the published rat cDNA (bp 4337-4355) was found to be absent from the mouse gene. This altered the reading frame of the mouse periaxin until base 4376 was reached, at which point an additional nucleotide returned the reading frame to that seen in the rat periaxin.

A.

```
MOUSE     1 MEARSRSAEE LRRAELVEII VETEAQTGVS GFNVAGGGKE GIFVRELRED SPAAKSLSLQ EGDQLLSARV FFENFKYEDA LRLLQCAEPY KVSFCLKRTV
RAT       1 ********** ********** ********** ********** ********** ********** ********** ********** ********** **********

MOUSE   101 PTGDLALRPG TVSGYEMKGP RAKVAKLNIQ SLAFVKKKKM VTGALGTPAD LAFVDVEFSF PKFSRLPRGL KAEAVKGFVP AAPARRRLQL PRLRVREVAE
RAT     101 ********** ********** ********** **S******* *I*T******* ********** ********** **D******* ********** **********

MOUSE   201 EAQVARMAAA APPPRKAKAE AEAATGAGFT APQIELVGPR LPSAEVGVPQ VSVPKGTPST EAASGFALHL PTLGLGAPAA PAVEPPATGI QVPQVELPTL
RAT     201 ********** ***S****S* **V******* ********** *********K ********** ********** ********** ******T*** **********

MOUSE   301 PSLPTLPTLP CLDTQEGAAV VKVPTLDVAA PSMGVDLALP GAEVEAQGEV PEVALKMPRL SFPPRFGIRGK EATEAKVVKG SPEAKAKGPR LRMPTFGLSL
RAT     301 ********** ********** ********** **VE****** ********** ********** ******V*** ********** ********** **********

MOUSE   401 LEPRPSGPEA VAESKLKLPT LKMPSFGIGV AGPEVKAPTG PEVKLPKVPE VKLPKVPEAA IPDVQLPEVQ LPKMSDMKLP KIPEMVVPDV RLPEVQLPKV
RAT     401 **S******V A********* ********S* ********K* ********** I****A**** ********** ********** *****A**** H****K****

MOUSE   501 PEMKVPEMKL PKWPEMAVPD VHLPDVQLPK VPE-----MK LPKVPEMAVP DVHLPDVQLP KVPEMKLPEM KLPKVPEMAV PDVRLPEVQL PKVSEVKLPK
RAT     501 ********** **I******* *****I**** ***MKLPD** ********** ******I*** ********D* ********** ****I***** **********

MOUSE   596 MPEMAVPDVH LPELQLPKMS EVKLPKMPEM AVPDVRLPEV QLPKVSEMKL PKMPEMTMPD IRLPEVQLPK VPDIKLPEMK LPEIKLPKVP DMAVPDVPLP
RAT     601 I*D******R ********** ******I*D* ********** *******L** **V******* ********** *****----- ********** E*********

MOUSE   696 ELQLPKVSDI RLPEMQVSQV PEVQLPKMPE MKLSKVPEVQ RKSAGAEQAK GTEFSFKLPK MTMPKLGKVG KPGEASIEVP DKLMTLPCLQ PEVGTEASHV
RAT     696 ******---- ------**** ***H***V** ***P****A* *********E K********** **V******T *****G**** ***LI***** ******VAR*

MOUSE   796 GVPSLSLPSV ELDLPGALGL EGQVQEAVPG KVEKPEGPRV AVGVGEVGFR VPSVEIVTPQ LPTVEVEKEQ LEMVEMKVKP SSKFSLPKFG LSGPKAVKGE
RAT     786 ********** ********** ********S* ********** ***T**A*** *******N** ******K*** ********** T********* ********A*

MOUSE   896 VEGPGRATKL KVSKPTISLP KARAGTEAEA KGAGEAGLLP ALDLSIPQLS LDAQLPSGKV EV--ADSKPK SSRFALPKFG VKGRDSEADV LVAGEAELEG
RAT     886 ********** *****A**** R*****D*D* ********** ********** ********** **AG*E**** G********* A********* **********

MOUSE   994 KGWGWDGKVK MPKLKMPSFG LSRGKEAETQ DGRVSPGEKL EAIAGQLKIP AVELVTPGAQ ETEKVTSGVK PSGLQVSTTG QVVAEGQESV QRVSTLGISL
RAT     986 ********** ********** ********I* ********** ********** E********* ********** *********R ********GA ****S*****

MOUSE  1094 PQVELASFGE AGPEIVAPSA EGTAGSRVQV PQVMLELPGT QVAGGDLLVG EGIFKMPTVT VPQLELDVGL GHEAQAGEAA KSEGGIKLKL PTLGTGSRGE
RAT    1086 ********** *****A**** ***V***I** ********** ********** ********** ********** ********T* *****L**** ****A*GK**

MOUSE  1194 GVEPQGPEAQ RTFHLSLPDV ELTSPVSSHA EYQVVEGDGD GGHKLKVRLP LFGLAKAKEG IEVGEKVKSP KLRLPRVGFS QSESVSGEGS PSPEEEEEGS
RAT    1186 *A*A*S**** H***I***** ********** ********** ********** *****R**** **T******* ********** ****A***** **********

MOUSE  1294 GEGASSRRGR VRVRLPRVGL ASPSKVSKGQ EGDATSKSPV GEKSPKFRFP RVSLSPKARS GSRDREEGGF RVRLPSVGFS ETAVPGSTRI EGTQAAAI*
RAT    1286 *****G**** ********** *****G**** ****A***** ********** ********** **K******* ********** ***A***A** *********
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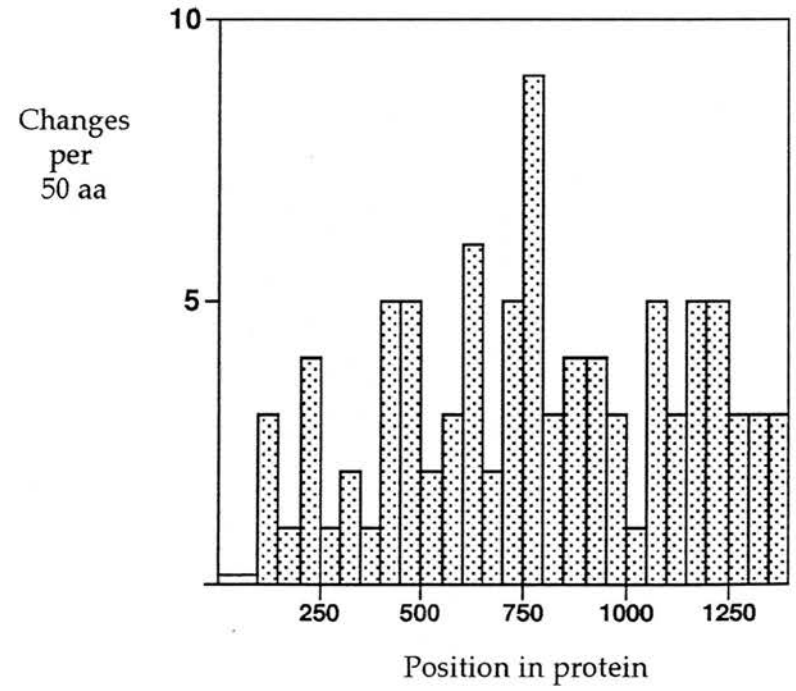
B.



PCRs carried out with primers spanning this region using S129 genomic DNA or a reverse transcription of sciatic nerve RNA from 15-day-old mouse as template, generated products which, when subcloned and dideoxy sequenced, were identical to the corresponding region of the mouse gene, therefore confirming the genomic clone sequence. A repeat of these experiments using rat genomic DNA or a reverse transcription of sciatic nerve RNA from 15-day-old rat as template for the PCR generated identical products which were structurally, much closer to the mouse gene than the published rat periaxin sequence, in that the 19 bp were absent and an extra bp was present at position 4376. Thus, these independent sources all appear to indicate that the published rat periaxin cDNA sequence is incorrect.

3.3 ANALYSIS OF THE 5' END OF THE MOUSE PERIAXIN GENE

3.3.1 The Mouse Periaxin Gene has a Single Transcriptional Initiation Site

A comparison of the sequences of several mouse periaxin cDNA clones obtained by 5' RACE showed that their 5' ends all terminated within a few nucleotides of each other and similarly within a few nucleotides of the 5' end of the published rat periaxin cDNA (Gillespie et al, 1994). To define accurately the transcriptional initiation site of the mouse *periaxin* gene, primer extension was performed. A ^{32}P-labelled oligonucleotide complementary to nucleotides 50-71 of the rat periaxin cDNA was hybridized with trigeminal nerve total RNA from 13-day-old mice, and this primer was extended with reverse transcriptase. The size of the products was determined by denaturing gel electrophoresis and autoradiography (Figure 20).

A single major extension product of 75 nt was thus demonstrated, mapping the transcriptional initiation site to 4 bp upstream of the putative exon 1. The possibility that these 4 extra bp are not part of the putative exon 1 but actually

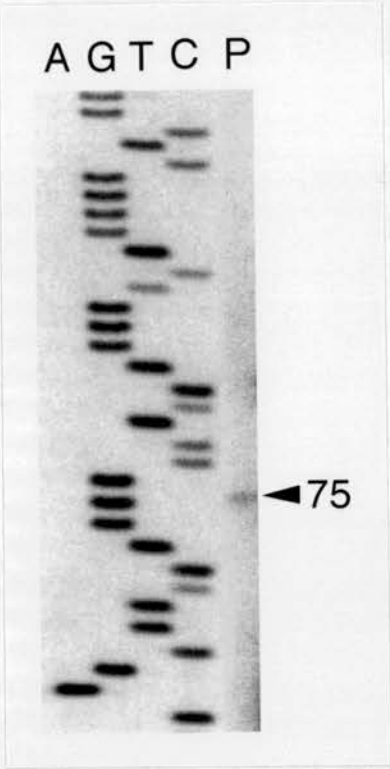


Figure 20. Determination of the periaxin transcription initiation site by primer extension. An oligonucleotide complementary to nts 50-71 of rat periaxin cDNA (Gillespie et al, 1994) was end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Total RNA (10 μg) from the trigeminal nerves of 13-day-old mice was hybridized to labelled oligonucleotide (5 pmol) and reverse transcribed. Extended products were resolved on a 6% polyacrylamide sequencing gel adjacent to a sequencing ladder comprising the 5'-end of periaxin primed using an oligonucleotide complementary to nts 55-76 of mouse periaxin cDNA. The extended product for murine periaxin mRNA is shown in lane P.

form a separate exon is extremely remote. Further evidence against this is indicated by the upstream gene sequence, which shows that the transcriptional initiation site would correspond to an A residue of a CA dinucleotide, at which many eukaryotic RNA polymerase II transcripts are observed to initiate (Corden et al, 1980)

3.3.2 The Murine *Periaxin* Promoter is TATA-less

Approximately 500 bp upstream of the deduced transcription inititation site was sequenced and is shown in Figure 21. Several features of this region are of interest. First, this putative core promoter does not contain a conventional TATA box and hence belongs to the family of TATA-less promoters. Such promoters are found mainly in 'house keeping' genes, which are genes expressed in a number of tissues at low levels. Other features of such promoters include (i) multiple transcriptional initiation sites, (ii) the absence of a canonical CAAT box and (iii) a high G+C content (due in part to the presence of multiple binding sites for the ubiquitous zinc finger transcription factor Sp1 (GGGCGG)). The mouse *periaxin* promoter possesses none of these features even though it is TATA-less: (i) by primer extension only a single major transcriptional intiation site is demonstrated; (ii) a CAAT box consensus sequence is located from -86 to -76; (iii) the sequence is only slightly G+C rich overall (54%) with no Sp1 binding sites present. This lack of many of the features of house keeping gene promoters is unsurprising given the very limited expression pattern of periaxin (Gillespie et al, 1994; Scherer et al, 1995).

An interesting feature of the G and C content of the *periaxin* promoter is that it is very deficient in the dinucleotide 5' CG 3'. By random pairing, the expected number of CG dinucleotides should be between 6-10 in every 100 nt. For the *periaxin* promoter this value is 10 x lower at 0.6 CG dinucleotides per 100 nt. Such suppression of CG pairs has been linked to transcriptional repression by methylation (Bird, 1987; Busslinger et al, 1983; Yisraeli et al, 1986), and indeed this

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-491  ACAAC CTTCA AGAGG TAAGC ATTCT TACTA CTACT ATTTC AAAGA CAAAG
                                                    STATx
-441  AACTG AGGGC CCAGT GATGC CCTGT GATTG CTGAA GTTCC CGGAG CCAGG

-391  AATGC CTGAG CTGGG ACTTG AACCC AAATT CTCAA GGAGA CAGTG TAAGA
                                  GCRE
-341  GGTTG TTTTG AGAGT CTCAG GGACT ATGTG GGCTA GGCCT GGGTC CAGGA
                               SREBP-1
-291  GCCCA GAGAT GATGG GGGTG GGGTG CTGAG TATTC GAATG GGGGT GGTAT
      SCIP/Oct-6
-241  GCAAA TTCTT GAACT AAGGC TGGGG TGGAG GTCTG GGCTC TGGCA AGGGA

-191  GAGGT TCATG TTTCC ACATC CCCAC GCCCT TGCAG ATGGA TGTAG TGCAC

-141  AGAAG GGGAT GTTTG TTTCC CCACC ACCCA CCCTT TGGGT GACAT CACAG
            NF-Y/CAAT
 -91  CAGGA CAGCC AATGG CCAGG GTCCC CCTTC TGCCC CCCCC TTCCC ATTCC
                                                    +1
 -41  TTTTC CCTCC CAGCC CAAGG CTAGG ACTAA CAGAA CAGAC CAAGG AGCTC

 +10  TGGAG GTGTC TGGAG GCCCA CCGgt
```

Figure 21. Nucleotide sequence of the mouse periaxin core promoter. Exon 1, which comprises 32 bp (positive numbers), and 491 bp of the putative core promoter (negative numbers) are shown relative to the position of the transcriptional initiation site (+1) determined by primer extension. The sequences recognized by STAT proteins (Horvath et al, 1995), SCIP/Oct-6 (Jaegle et al, 1996; Bermingham et al, 1996; Blanchard et al, 1996), SREBP-1 (Kim et al, 1995) and NFY (Hooft van Huijsduijnen et al, 1987) together with the concensus glial cAMP response element (GCRE) (Li et al, 1994) are underlined The CAAT box is double underlined. Sequences common to myelin gene promoters (but of unknown function) are underlined with a dotted line.

may be a very important mechanism for the tissue-specific and stage-specific regulation of the *periaxin* gene.

An extensive comparison of the core promoter sequence with published transcription factor binding sites (Faisst and Meyer, 1992; TFSEARCH web site, Kyoto University (www.genome.ad.jp/SIT/TFSEARCH.html)) identified several potential elements. A number of the elements bind ubiquitous factors (NF-Y, SREBP-1, STATx)(Horvath et al, 1995; Hooft van Huijsduijnen et al, 1987; Kim et al, 1995); such factors are probably required for basal levels of transcription, acting co-operatively with tissue-specific and stage specific factors. Of particular interest with respect to myelination is the presence of an octamer motif (ATGCAAAT) between –236 and –243. This is a potential binding site for the POU family of transcription factors which often play an important role in cell-type specific transcriptional regulation (Schöler, 1991; Ruvkun and Finney, 1991; Wegner et al, 1993). The only members of this family present in Schwann cells are Oct-1 and SCIP/Oct-6 and of these only SCIP/Oct-6 is differentially regulated during Schwann cell development (Kuhn et al, 1991; Blanchard et al, 1996; Monuki et al, 1989) and indeed this protein has recently been shown to be a vital factor for the maturation of Schwann cells from a premyelinating to a promyelinating phenotype (Blanchard et al, 1996; Bermingham et al, 1996; Jaegle et al, 1996). Similarly, between -317 and -306 the sequence shows a high degree of homology to the GCRE element present in the promoters of several myelin genes where it is believed to mediate their induction in response to the increased intracellular levels of cAMP which result from the addition of forskolin to Schwann cell primary cultures (Li, et al, 1994). This effect is believed to mimic some of the axonal signals required for Schwann cell differentiation and may indicate a concerted pathway for the activation of myelin gene expression (Lemke and Chao, 1988; Trapp et al, 1988). The identification of this element, together with a number of other sequences commonly found in myelin gene promoters, and the shared CG suppression, would

appear to suggest that *periaxin* is regulated by the same pathway, underlining its potential importance in the myelination process.

3.4 ANALYSIS OF MOUSE PERIAXIN GENE EXPRESSION

In postnatal rats, the *periaxin* gene is believed to be expressed exclusively in the PNS as two distinct mRNAs of similar abundance (Gillespie et al, 1994). The amounts of these mRNAs reflect the activity of Schwann cell myelin deposition, given that they are seen to parallel those of the major myelin proteins in normal sciatic nerves and in those subjected to axotomy and crush (Scherer et al, 1995; Lemke and Axel, 1985). The spatial and temporal expression of the *periaxin* gene in the mouse was thus examined. Total RNA from various tissues of a 15-day old mouse was electrophoresed, transferred to nylon membrane and probed with a ^{32}P-labelled restriction fragment corresponding to nts 1-1323 of the 4.6 kb rat periaxin cDNA (Gillespie et al, 1994).

As can seen in Figure 22, two distinct periaxin mRNAs are detected, specifically in the PNS. This is in complete agreement with the previous findings in the rat. Using the same probe as for the tissue Northern blot, the developmental expression of these mRNAs in postnatal mouse sciatic nerve (PNS) was investigated (Figure 23). Both periaxin mRNAs are detected in significant quantities in newborn animals, increasing to a peak at P16-P18. Interestingly, this high level of expression is maintained into adulthood (although the 5.2 kb mRNA appears to slightly downregulate).

Given that the mouse is approximately 2 days more advanced, developmentally, than the rat, the early postnatal expression of periaxin (during the most active phase of myelin deposition) is reasonably similar in both species. However, at P21 in the rat the levels of periaxin mRNA are seen to drastically

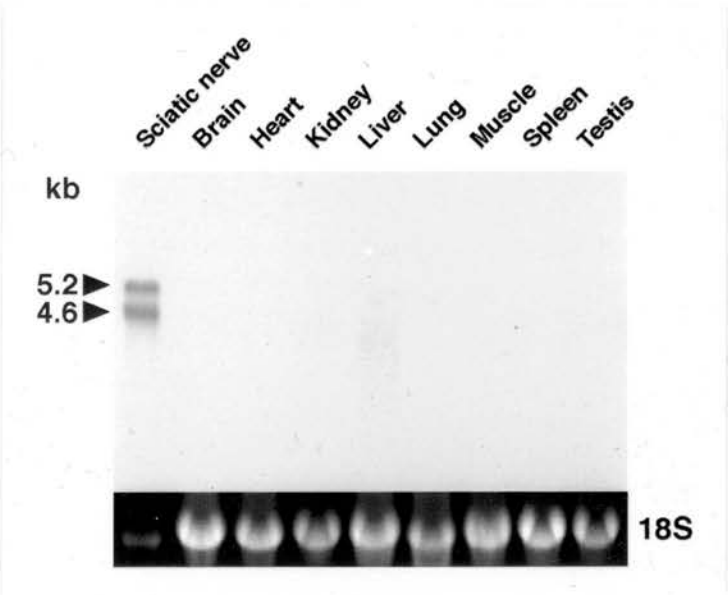


Figure 22. The mouse periaxin gene is specifically expressed in the PNS. Total RNA from various tissues of a 15-day-old mouse was electrophoresed on a 0.8% agarose formaldehyde gel (2 μg per lane; except sciatic nerve RNA, 0.5 μg), transferred to nylon membrane and probed with a 1.3 kb restriction fragment corresponding to bases 1-1324 of the published rat periaxin sequence, labelled with [α-^{32}P]dCTP by random priming. Tissues are indicated at the top of the figure, and the ethidium bromide-stained 18S RNA in each lane is shown in the bottom panel to show the similar RNA loadings. Periaxin is detected as two distinct mRNAs (of approximately equal abundance) in the sciatic nerve only.

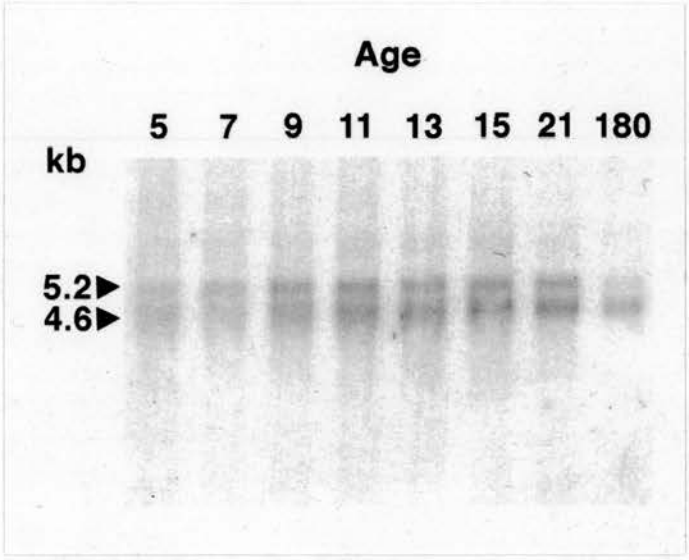


Figure 23. Mouse periaxin mRNAs are developmentally regulated. Sciatic nerve RNA from mice of various ages was electrophoresed on a 0.8% agarose formaldehyde gel (2 μg per lane), transferred to nylon membrane and probed with a 1.3 kb restriction fragment corresponding to bases 1-1324 of the published rat periaxin sequence, labelled with [a-^{32}P]dCTP by random priming. Postnatal ages (in days) are indicated at the top of the figure The levels of both periaxin mRNAs reach a peak between 2-3 weeks after birth, after which they decline very slowly, a feature which is more pronounced for the 5.2 kb message.

decline and are almost undetectable in the adult. This is in total contrast to the mouse where levels remain elevated throughout the life of the animal. The reason for this difference in the temporal pattern expression between such closely related species is unclear. It is possible that other sciatic nerve mRNAs may be increasing more in the rat than in mouse, so that total RNA will contain proportionately less periaxin mRNA per µg. Indeed, there are still reasonable levels of periaxin protein in the sciatic nerves of adult rats indicating continued gene expression (Scherer et al, 1995). The polarization of expression within these nerves from an adaxonal localization in young animals to an abaxonal localization in the adult would seem to suggest that periaxin has a long term role which therefore requires long term expression.

3.5 ALTERNATIVE SPLICING OF THE MOUSE PERIAXIN GENE

3.5.1 Characterization of Periaxin mRNA structure

As *Prx* is a single copy gene in both rat and mouse (this thesis; Gillespie et al, 1994, 1997), it follows that the two mRNAs must be generated from this single gene and must thus be products of alternative splicing. For the previously characterized 4.6 kb periaxin mRNA, splicing of the primary gene transcript results in the incorporation of all seven exons shown in Figure 16. Therefore, to characterize the exonic structure of the 5.2 kb mRNA, various possible alternative splicing mechanisms (Breitbart et al, 1987) were investigated.

Northern blotting carried out on rat and mouse sciatic nerve total RNA with regional probes spanning the entire 4.6 kb mRNA showed strong hybridization to both messages (not shown). A particularly important implication from this was that exon 7 is also present within the 5.2 kb mRNA and thus at least 86% of the 4.6 kb mRNA (and more than 91% of the protein coding sequence) is shared with the larger

message. The detection by Gillespie et al (1994) of only a single translated periaxin protein from the two periaxin mRNAs of rat appeared to confirm this finding, suggesting that the two mRNAs contain the same coding region and therefore encode the same 147 kDa protein. The size difference of the mRNAs was thus presumed to be most likely due to the presence of a longer untranslated sequence in the larger message.

The 3' UTR of the 4.6 kb mRNA is defined by the stop codon (TGA) and the start of the poly(A) tail and measures 210 and 205 bp in the mouse and rat respectively. For the 5.2 kb mRNA to possess a longer 3' UTR the polyadenylation signal utilized in the 4.6 kb mRNA should be ignored in favour of a second signal further downstream. Analysis of approximately 800 bp of mouse genomic clone sequence directly downstream of the known polyadenylation signal failed to identify a second polyadenylation signal. Also, a PCR product generated to this sequence and used as a probe for Northern blot of mouse sciatic nerve RNA failed to identify the 5.2 kb message. It was thus concluded that the 3' ends of the 4.6 and 5.2 kb periaxin mRNAs are identical and that the difference between the two messages must be located at a position somewhere 5' of exon 7 ie involve alternative splicing of exons 1-6.

3.5.2 Two Periaxin mRNAs are Generated by a Retained Intron Mechanism

Using a probe encoding nts 1-599 of the 4.6 kb rat periaxin cDNA that had been previously shown to hybridize to both mRNAs on a Northern blot of sciatic nerve RNA from 15-day-old rat, a rat sciatic nerve cDNA library was screened for clones corresponding to the 5' end of each periaxin mRNA. From the 3×10^5 library bacteriophage that were screened, 18 clones were identified and isolated. Characterization of each clone by dideoxy sequencing and PCR using exon-specific primers showed 13 of the clones to be identical in structure to the 5' end of the 4.6 kb mRNA ie these clones possessed no extra sequence relative to this mRNA. The

sequence of the 5 other clones was also identical to the 5' end of the 4.6 kb mRNA but only up to the end of sequence corresponding to exon 6. After this point, the sequence of these clones showed great similarity to that of intron 6 of the murine gene. Although 3 of these clones ended within this sequence, 2 extended through 597 bp, at which point the clones continued into sequence corresponding to the start of exon 7 of the 4.6 kb mRNA, which strongly suggested that the 5.2 kb periaxin mRNA differed from the 4.6 kb mRNA by the non-splicing of the 0.6 kb intron located between exons 6 and 7 of the gene. To confirm this finding, sciatic nerve RNA from 15-day-old mice was Northern blotted with a probe corresponding to the 'intronic' sequence from one of the above 5.2 kb-specific clones which had been generated by PCR (Figure 24). The hybrization of this probe to the 5.2 kb periaxin mRNA shows that the 'intron' is indeed present within this message.

Further confirmation of this finding can be seen from the screen itself. From the similar abundance of the two periaxin mRNAs displayed by Northern blotting, the library screening would be expected to identify similar numbers of 4.6 kb and 5.2 kb-specific clones. At first glance it would appear that almost three times as many 4.6 kb-related clones were isolated in comparison to those of the 5.2 kb. However, of the 13 clones identical in structure to the 5' end of the 4.6 kb mRNA only 6 actually extended far enough to show that they did not include this 'intron' sequence. The remaining 7 clones encoded only exons 1-6 and could therefore actually be from either the 4.6 kb or the 5.2 kb mRNA. Thus, only 6 clones were shown to be specifically related to the 4.6 kb mRNA compared to 5 specific to the 5.2 kb mRNA - an almost 1:1 relationship.

The deduced structural relationship of the two periaxin mRNAs and the murine gene, is shown in Figure 25. As indicated, the 4.6 kb periaxin mRNA is generated by constitutive splicing of the primary gene transcript, with the removal all 6 introns and the splicing of each of the 7 exons. The 5.2 kb mRNA is an alternatively spliced variant of the same primary gene transcript from which only

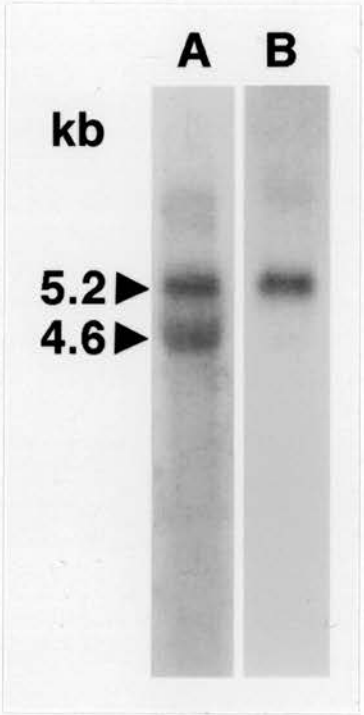


Figure 24. Alternative splicing of an intron generates the two periaxin mRNAs. Sciatic nerve RNA from 15-day-old mice was electrophoresed on a 0.8% agarose formaldehyde gel (2 μg per lane), transferred to nylon membrane and probed in lane A with a 1.3 kb restriction fragment corresponding to bases 1-1324 of the rat periaxin cDNA and in lane B with a PCR product comprising intron 6 of the periaxin gene. Intron 6 is shown to be specific to the 5.2 kb mRNA.

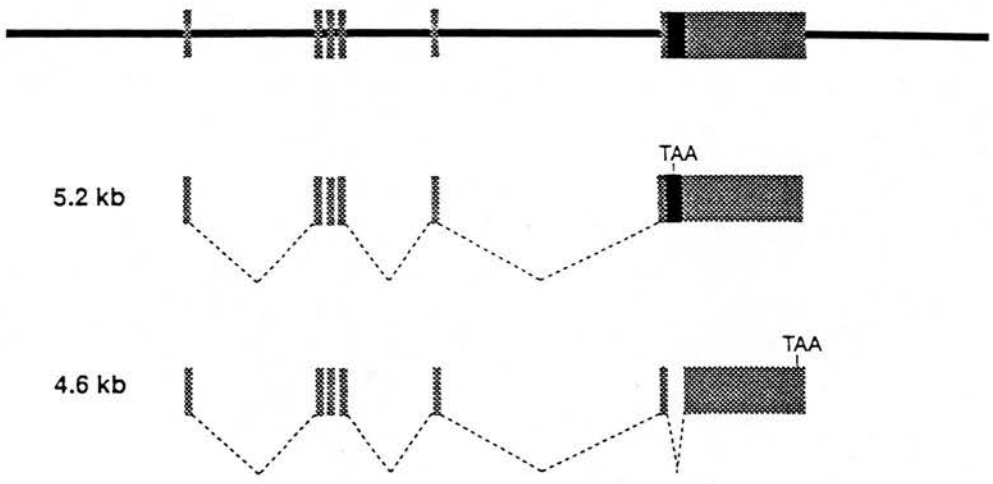


Figure 25. Processing of the periaxin gene primary transcript. Exons present in the final processed mRNA are represented by filled boxes. Sequences removed (spliced) from the primary gene transcript are shown as dotted lines.

the first 5 introns are removed. The resulting message not only combines all 7 exons as in the 4.6 kb mRNA, but also retains the 0.6 kb intron located between exons 6 and 7.

3.5.3 The 5.2 kb Periaxin mRNA encodes a Truncated Isoform

Given that the 4.6 kb mRNA encodes the previously described 147 kDa periaxin protein (Gillespie et al, 1994) and that it does so from an open reading frame which spans from exon 4 to the extreme 3' end of exon 7, it follows that the position of the retained intronic sequence within the identical coding region present in the 5.2 kb, must affect any protein translated from this message. Subsequent analysis of the open reading frame present in the 5.2 kb sequence, assuming the same initiation codon is used in the translation of the 4.6 kb mRNA, indicated that a highly truncated periaxin isoform would be produced due to the presence of a stop codon located 63 nts into the retained intron which extends exon 6. This deduced protein would be identical to the N-terminal 127 amino acids of the 147 kDa periaxin but would diverge into a novel 21 amino acid, intron-encoded C-terminus, giving a molecular weight of just over 16 kDa.

To rule out the possibility that the intron-derived stop codon was a sequencing error and that the retained sequence was actually translated throughout and in phase with exon 7, rat periaxin cDNAs corresponding to the 4.6 kb mRNA (pA1BG; Gillespie et al, 1994) and the 5.2 kb mRNA (constructed by replacing the SfiI/HincII restriction fragment of the pA1BG periaxin cDNA with the corresponding fragment - which included the entire intron sequence - from one of the 2 long, 5.2 kb-specific cDNA clones isolated above) were transcribed *in vitro*, generating RNAs which were subsequently translated by rabbit reticulocyte lysate in the presence of [^{35}S] L-methionine. Figure 26 shows the resulting gel autoradiograph following electrophoresis of these translated proteins. The 4.6 kb cDNA encodes a large protein with a M_r equivalent to that demonstrated by

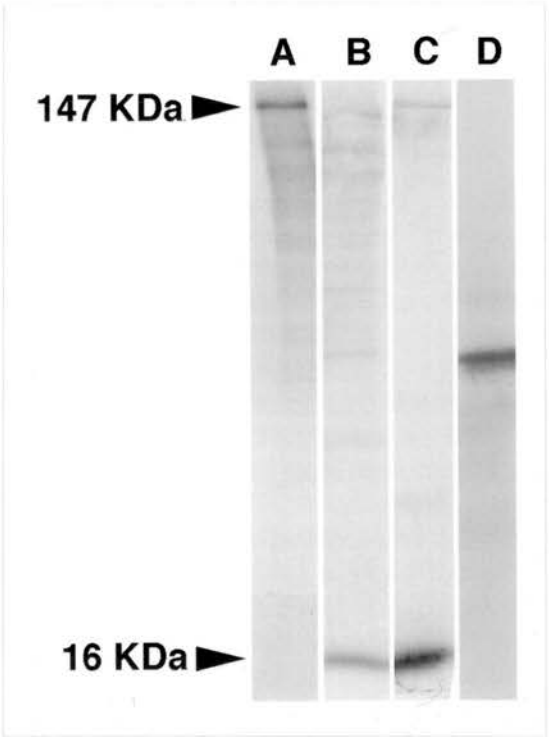


Figure 26. Transcription-translation and immunoprecipitation of the L- and S-periaxin isoforms. cDNAs corresponding to the 4.6 and 5.2 kb rat periaxin messages were transcribed with T7 RNA polymerase then translated with rabbit reticulocyte lysate in the presence of [^{35}S]-labelled L-methionine. A sample of each was then electrophoresed on a 4-20% linear gradient PAGE gel. After drying, the gel was exposed to X-ray film for 48 h. Lanes A and B represent the transcription/translation products of the 4.6 and 5.2 kb cDNAs respectively. A mixture of these two reactions and a control translation product (ELAV; a gift from Dr S. Tait) was subsequently immunoprecipitated with the anti-NTerm antibody. Electrophoresis and autoradiography was as before. Lane C demonstrates that the NTerm antibody specifically immunoprecipitates the L- and S-periaxin proteins even when other proteins are in excess (lane D; ELAV).

Gillespie and coworkers (1994) in the transcription-translation of the same clone. In contrast, the 5.2 kb cDNA encodes a much smaller protein (approximately 16 kDa). This correlates well with the Mr predicted from the sequence translation of the 5.2 kb mRNA. To further verify that this small protein encodes the predicted sequence ie that it has been translated from the same initiating methionine as the 147 kDa periaxin and is not an artefact of a misprimed translation from another position on the 5.2 kb RNA, a quantity of the *in vitro* translation was immunoprecipitated with an antibody raised to the extreme N-terminus of the large periaxin (anti-NTerm). As Figure 26 shows, this antibody does indeed precipitate the 16 kDa protein, thus confirming it as a truncated isoform of periaxin.

Although these experiments demonstrated a truncated form of periaxin could be generated from the 5.2 kb mRNA, no such protein has been detected by previous studies (Gillespie et al, 1994; Scherer et al, 1995). However, given these studies both utilized the anti-p170 antibody which recognizes sequence within the repeat region of the 147 kDa periaxin protein and given the truncated periaxin isoform is predicted not to encode any of the repeat domain, this is unsurprising. Actual expression of this isoform was demonstrated *in vivo* by Western blots carried out on sciatic nerve homogenate from 15-day-old mouse using the anti-Nterm and anti-170pep1 antibodies as well as a rabbit anti-peptide antibody raised against the unique 21 amino acid C-terminus of the small periaxin isoform (anti-SPeri; Figure 27). Levels of the small periaxin isoform (subsequently named S-periaxin) can be seen to be remarkably high relative to those of the large periaxin isoform (subsequently named L-periaxin) and are probably consistent with the relative levels of the mRNAs. The possibility that the S-periaxin band is actually a degradation product cannot be completely discounted given the extreme susceptibility of the 147 kDa periaxin to proteolysis (Gillespie et al, 1994). However, the presence of a cocktail of proteinase inhibitors throughout the procedure together with the fact that the S-periaxin is the only other band and that

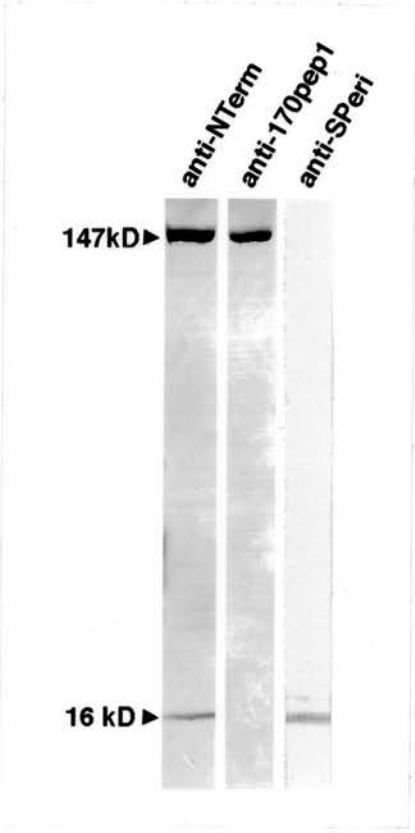


Figure 27. Western blot of mouse periaxin with domain-specific antibodies. Sciatic nerves from 15-day-old mice were homogenised and immunoblotted using antibodies raised against peptide sequences comprising either the N-terminus of L- and S-periaxin (anti-NTerm), the repeat region unique to L-periaxin (anti-170pep1; Gillespie et al, 1994) or the C-terminus unique to S-periaxin (anti-SPeri). The sizes of L- and S-periaxin are indicated in kDa.

it is also of the predicted size, makes this very unlikely. Similarly, the absence of an S-periaxin-size band on a Western blot carried out on purified myelin with the anti-NTerm antibody and under the same procedural conditions (not shown) would suggest that proteolysis of the L-periaxin has not occurred. This finding, that S-periaxin is not present within, or does not co-localize with myelin (unlike the L-periaxin isoform) suggests a different function for this isoform during development of the PNS. This differential localization was also supported by immunocytochemical analysis (see section 3.6)

3.5.4 A Possible PDZ Domain at the N-terminus of L- and S- Periaxin

A comparison of the deduced amino acid sequence of both L- and S-periaxin between rat and mouse (Figure 28) demonstrated a particularly striking similarity between the N-terminal 127 amino acids up to the point where the isoforms diverge. Within this region the rat and mouse polypeptides are identical. Given that the percentage similarity for the rest of L-periaxin is 94% (6 changes per 100 aa), with the next largest run of identical sequence only 50 amino acids long, the N-terminus of both isoforms appears to be highly conserved. It was thus postulated that this region must possess some important motifs for the function of periaxin, prompting a careful examination of the sequence, in spite of the fact that previous searches of the complete L-periaxin polypeptide had not been informative (Gillespie et al, 1994). A BLAST search (Macvector, Oxford Molecular Group) using the deduced amino acid sequence for S-periaxin revealed a short region of similarity to the tight junction-associated protein, zonula occludens (ZO-1) (Willott et al, 1993; Itoh et al, 1993; Woods and Bryant, 1993). This region was later identified as a PDZ domain (D. Sherman, personnal communication), a motif found in a number of proteins which are believed to associate with the plasma membrane (Fanning et al, 1996; Kornau et al, 1995; Takeuchi et al, 1997). This is very interesting given L-periaxin

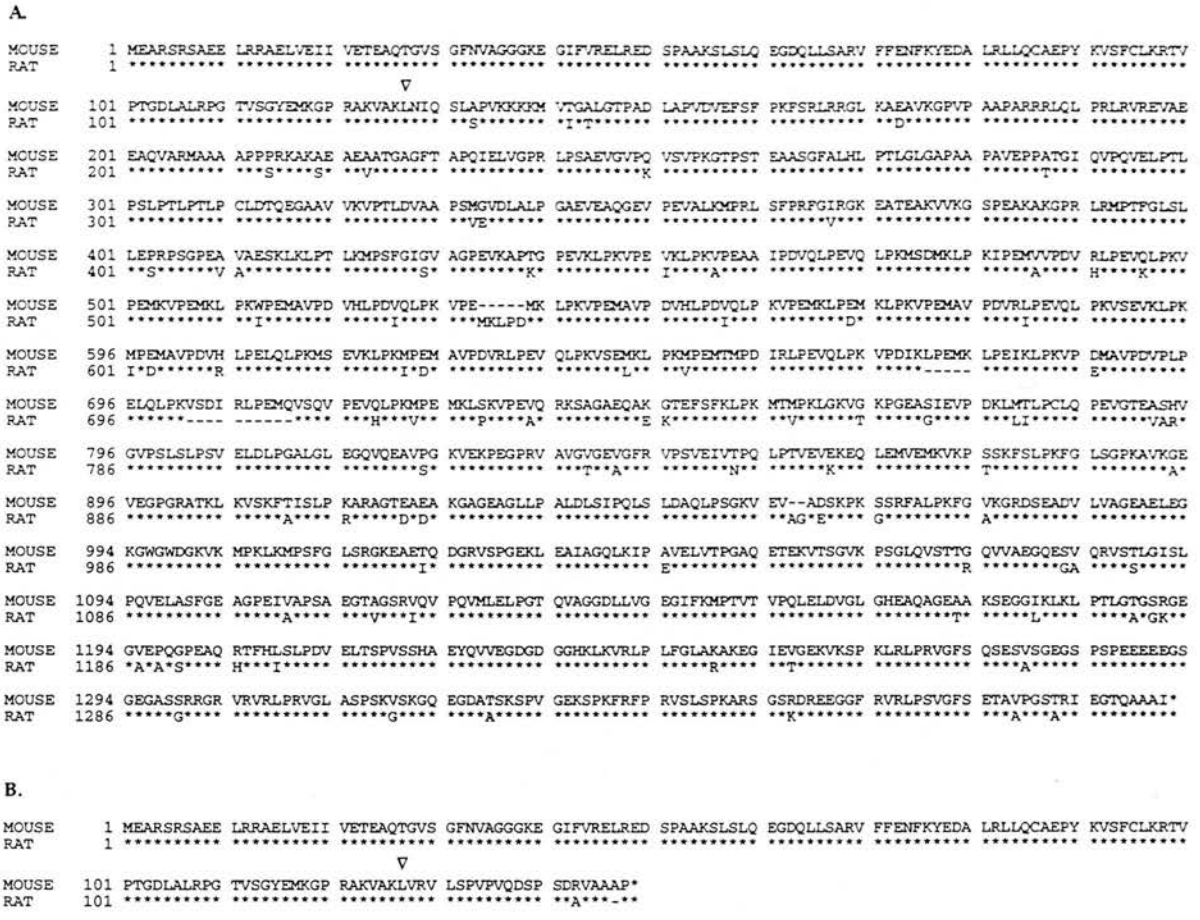


Figure 28. Interspecies comparison of the deduced amino acid sequences of the large (A) and small (B) periaxin isoforms. Conserved amino acids are represented by *asterisks*. *Dashes* indicate nucleotide gaps introduced for optimal alignments. The point of sequence divergence between the two periaxin isoforms is arrowed (∇).

has been shown to interact with the Schwann cell plasma membrane in the rat (Scherer et al, 1995).

3.6 SUBCELLULAR LOCALIZATIONS OF S- AND L- PERIAXIN WITHIN MYELINATING SCHWANN CELLS

3.6.1 L- and S-Periaxin Show Contrasting Localizations in Myelinating Schwann Cells

To compare the subcellular localizations of the two periaxin isoforms, thin transverse sections of 20-day-old mouse sciatic nerve were immunofluorescently double-labelled for L-periaxin and MBP, and for S-periaxin and MBP. On sections double-labelled for L-periaxin and MBP (Figure 29; A-C), bright L-periaxin immunoreactivity was visible at the plasma membrane and both the abaxonal and adaxonal (periaxonal) surfaces of the Schwann cells but not within compact myelin (as defined by red-fluorescent MBP labelling). Sections double-labelled for S-periaxin and MBP (Figure 29; D-F) show bright S-periaxin immunoreactivity within Schwann cells showing the same strong fluorescent MBP labelling of compact myelin but for this isoform the localization is exclusively cytoplasmic, with no plasma membrane or abaxonal or adaxonal staining.

The subcellular localization pattern shown here for mouse L-periaxin is very similar to that previously found for the protein in rat sciatic nerve (Scherer et al, 1995; Gillespie et al, 1994). Using the same L-periaxin isoform-specific antiserum as this work, these investigations identified predominant labelling of the plasma membrane and abaxonal surface of Schwann cells possessing mature myelin. Adaxonal (periaxonal) immunoreactivity was also visible in these cells but at a much lower level however. In young or regenerating nerves the opposite was shown, with periaxonal labelling predominating over reduced levels of plasma membrane and abaxonal immunoreactivity. It is important to note that in these studies,

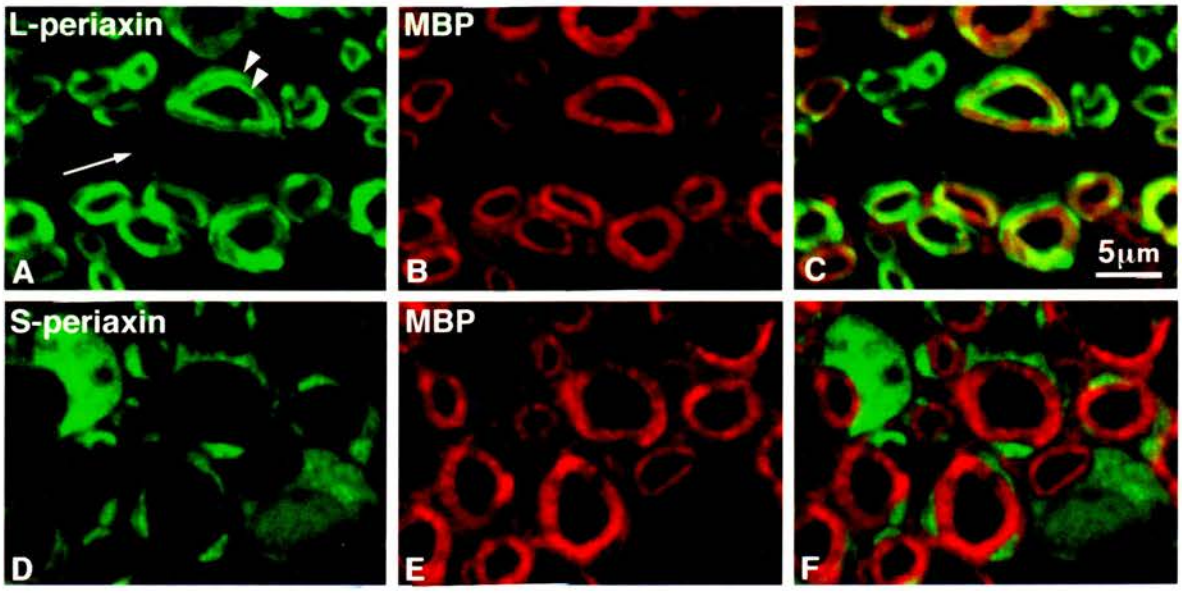


Figure 29. Indirect immunofluorescence confocal micrographs showing the subcellular localization of large (L)- and small (S)-periaxin. (A-C) Transverse section of sciatic nerve from a 20-day-old mouse, immunofluorescently double-labelled for L-periaxin (A; fluorescein) and MBP (B; streptavidin-Texas red). The superimposed image is shown in C. L-periaxin can be seen to be exclusively localized to the plasma membrane (arrow) and both the abaxonal (apposing the basal lamina) and adaxonal (apposing the axon) surfaces (arrowheads) of myelinating Schwann cells. MBP labelleling is restricted to the myelin sheath and therefore shows no co-localization (which would appear as orange-yellow). (D-F) Transverse section from the same sciatic nerve preparation, double-labelled for S-periaxin (D) and MBP (E). The superimposed image is shown in F. S-periaxin distribution is shown to be restricted to the cytoplasm of myelinating Schwann cells with no membrane labelling and no co-localization to MBP.

immunoreactivity is simply classified as being that of 'periaxin' and not, more accurately, as being that of the 'L-periaxin isoform'. This is due to the fact, that at the time, only one periaxin protein was known. As the antibody used in these previous investigations was raised against a sequence found to be absent from the S-periaxin isoform, the immunoreactivity shown has therefore been accepted as being that of the L-periaxin isoform).

Discussion

4 DISCUSSION

In this study, the gene encoding periaxin in the mouse has been isolated and characterized and its expression analysed. It has been revealed that the gene not only encodes the previously described 147 kDa periaxin protein (Gillespie et al, 1994) but also encodes a truncated isoform of 16 kDa which is generated by a rare form of alternative splicing. Furthermore, these two proteins have been shown to be differentially targeted within myelin-forming Schwann cells, implying a dual role for periaxin.

4.1 Origin of the *Periaxin* Gene Structure

The mouse *periaxin* gene (*Prx*) is composed of 7 exons interrupted by 6 introns and spans approximately 20.6 kb. The *Prx* intron sizes (88 bp to 7500 bp) and the sizes of exons 1-6 (32 bp to 197 bp) are typical when compared to the size range of introns and exons in an average vertebrate nuclear gene (Hawkins, 1988). However, exon 7 is unusually long (4002 bp), as vertebrate coding exons are rarely over 800 bp in length. Indeed, it is this size limit which has added weight to the 'intron-early' hypothesis for the origin of split genes (Gilbert, 1978; 1987; Darnell and Doolittle, 1986). Naora (1987) and Senepathy (1986) have calculated that there is only a very low probability of an open reading frame generated from random nucleotide sequences exceeding 550-600 nt without encountering an in-frame stop codon. It has therefore been postulated that splicing has evolved to join such short open reading frames (exons) by removal of the sequences containing the stop codons (introns). Arguably, protein coding exons are not random sequences of nucleotides but, they must surely have originated from random processes. A small number of exons over this predicted length will obviously have arisen by chance but these will be a rare occurrence. The probability that the 4002 bp of *Prx* exon 7 arose in this way must be virtually nil.

It is sensible to assume that the *periaxin* gene must have evolved as multiple, short exonic sequences and that the introns separating these sequences have been lost. From the length of exon 7 it is possible that the ancestral *Prx* gene contained between 12-30 exons and therefore 5-23 introns must have been removed to generate the present gene structure. The most plausible explanation for the deletion of these introns is the homologous recombination of a cDNA generated by the action of a reverse transcriptase on a partially spliced intermediate periaxin mRNA. This mechanism has been well documented for the removal of introns from yeast genes (Fink, 1987) and has been used to explain the structural origins of number of vertebrate genes which are devoid of introns. However, the appearance of a characteristic A-rich tract at the 3' end of such genes (due to the addition of a poly(A) tail before splicing and reintegration of the transcript (Nevins and Cheng-Kiang, 1981) and lack of such a feature at the 3' end of *Prx* exon 7 raises the possibility that this long exon may have originated through the action of another unknown mechanism.

4.2 Alternative Splicing of the *Periaxin* Gene Involves Intron Retention

The *periaxin* gene is expressed during post-natal development as two distinct mRNAs of similar abundance. It has been shown here that the mRNAs are generated by alternative splicing of the primary gene transcript such that the last intron is retained in the larger (5.2 kb) mRNA but is lacking from the smaller (4.6 kb) mRNA. This form of alternative splicing in effect creates a larger fusion exon. With regards to the 5.2 kb mRNA, this means that exon 6, intron 6 and exon 7 become a single exon of approximately 4.8 kb encompassing more than 92% of the final transcript. Clearly, the distinction between intronic sequence and exonic sequence can be easily blurred - are not excluded 'exons' actually introns and are not retained 'introns' actually exons?

It has been shown that the factors determining the retention of introns are different from those responsible for the alternative splicing of exons. A key feature of intron retention (Dirksen et al, 1995) is the presence of suboptimal splice sites at both the 5'- and 3'-ends of the intron ie splice sites whose sequences differ from the consensus sequences that are known to promote splicing (Shapiro, 1987). Such 'weak' splice sites would not normally support splicing and are therefore dependent on additional enhancer elements located in the downstream exon (Dirksen et al, 1995). These exonic splicing enhancers (ESEs) are characteristically purine-rich and are postulated to function through interactions with transactivating factors (Dirksen et al, 1995).

In support of this model, the splice sites of the last intron of the *periaxin* gene are divergent from the consensus sequences and there is a domain downstream in exon 7 which is highly purine rich. The sequences of the 5'- and 3'-splice sites are CTGgtacgc and tcagG respectively which are significantly different from the corresponding rodent splice site consensus sequences of CAGgtragt and ncagG (Shapiro, 1987). Downstream of the acceptor site and 24 bases into exon 7 there is a 14 base sequence GAAGAAGAAGAAGA, which is an excellent candidate for an ESE (Dirksen et al, 1995).

This form of alternative splicing is even more complex when it is considered that intron-containing mRNAs, which are usually restricted to the nucleus, must now be transported to the cytoplasmic compartment. It has been proposed that splicing and nuclear export are competing pathways (Dirksen et al, 1995). The presence of either a strong 5' or 3' splice site kinetically favours the splicing pathway. Only when all introns have been spliced therefore will the fully processed mRNA be released for export. However, when both splice sites are weak, the pre-mRNA may form the splicing complex more slowly or weakly, favouring entry into the export pathway from the nucleus.

4.3 The *Periaxin* Gene Promoter May Bind Transcription Factors Which Regulate Myelination

Over the past decade there has been an explosion of interest in the regulatory elements involved in coordinating the expression of those genes required for myelination. Several key transcription factors have been identified together with an ever increasing number of common promoter sequences. Given that periaxin has been postulated to have a potential role in myelin deposition by Schwann cells (Gillespie et al, 1994) it was of considerable interest to examine the promoter region of the *periaxin* gene and place it in context.

A particularly intriguing finding was a binding site for the POU-domain transcription factor SCIP/Oct-6. This factor has been implicated as a major transcriptional regulator in Schwann cell development (Bermingham et al, 1996; Blanchard et al, 1996; Jaegle et al, 1996). As one of the first genes to be upregulated following axonal contact both *in vitro* and *in vivo* and responding similarly to chemicals which raise intracellular cAMP such as forskolin (Scherer et al, 1994), SCIP is well placed to regulate the expression of axonally dependent genes such as those of the myelin proteins and periaxin (Lemke et al, 1988; Trapp et al, 1988; Scherer et al, 1995), and indeed it has been shown to act as a negative repressor of both P0 and MBP expression (Monuki et al, 1993). However, the importance of a binding site for this factor within the *periaxin* promoter is questionable. In a homozygous SCIP null mutant mouse (Bermingham et al, 1996) in which most Schwann cells arrest at the promyelinating stage (where they have arranged themselves in a 1:1 manner but have not formed myelin), both periaxin and MAG are found around many of the axons where they are correctly targeted to the adaxonal surface. This would seem to suggest that SCIP is either not involved in the regulation of expression of these early genes and the initiation of myelination or, that it is perhaps replaceable.

Also of great interest is the presence of a sequence which bears considerable similarity to the consensus GCRE element found in several myelin protein genes and which has been shown to mediate induction in response to raised levels of intracellular cAMP (Li et al, 1994). This effect is believed to mimic some of the axonal signals required for Schwann cell differentiation and may be indicative of a common signalling mechanism for the activation of myelin gene expression (Lemke et al, 1988; Trapp et al, 1988). Given that axonal contact has been shown to be vital to maintain periaxin expression in Schwann cells *in vivo* (Scherer et al, 1995) and given that periaxin is believed to be the previously described protein, P170, which is known to be upregulated by 8-bromo cAMP in Schwann cells *in vitro* (Shuman et al, 1988), the identification of this element in the *periaxin* core promoter certainly suggests that periaxin is regulated by the same pathway as the myelin genes, underlining its potential importance in the myelination process.

4.4 The *Periaxin* Gene Promoter Shows Suppression of the Dinucleotide 5'CG 3'

The suppression of the dinucleotide 5' CG 3' within the putative *periaxin* core promoter is particularly interesting with respect to its tissue specific expression. Transcriptional repression by methylation is known to be a common mechanism for switching off genes in all but specific cell types or tissues (Yisraeli et al, 1986; Bird, 1987; Busslinger et al, 1983). Considerable evidence obtained from a large number of individual cell type-specific genes correlates their expression with the degree of undermethylation of their DNA sequences with the genes being highly methylated in non-expressing cells but considerably undermethylated in tissues in which they are expressed (reviewed in Yisraeli and Szyf, 1984). This is consistent with the idea that DNA methylation functions as a general repression signal for transcription and ensures gene inactivity in all tissues in which the gene should not be expressed. For every case analysed, methylation is present from early development with genes undergoing demethylation in a tissue-specific manner when they become expressed.

An example of this in a myelin gene can be seen with MAG (Konat, 1996). In the liver (non-expressing), the MAG genomic DNA is very heavily methylated. In contrast, most sites are demethylated in the brain. Similarly, MAG gene methylation is significantly less in mature oligodendrocytes than in their progenitors, indicating progressive demethylation concomitant with differentiation (Konat, 1996). Specific cell types must therefore contain specific proteins which recognise the gene in its methylated form and lead to its activation through demethylation.

Evidence for the presence of methylation within a promoter can come from the analysis of the number of CG dinucleotides. Methylation of the C residue of a CG dinucleotide can lead to accelerated rates of mutation as cytosine is prone to deamination and conversion to thymine when methylated (Bird, 1987). Therefore, CG dinucleotides tend to have been lost from sequences subject to methylation. The promoters of many cell type-specific genes are known to be CG deficient and methylation has therefore been proposed as a mechanism for regulating this form of expression. For instance, when a reporter gene linked to a methylated form of the CG-deficient α-actin promoter is transfected into fibroblasts, no expression is induced but, when transfected into myoblasts site specific demethylation is seen to occur, leading to expression (Yisraeli, 1986).

The promoters of most house keeping genes (which lack the TATA-box consensus) possess CG dinucleotides at a frequency predicted by random pairing (known as a CpG island) and indeed, it is the retention of these dinucleotides which may lead to the high G+C content of these promoters and the presence of multiple Sp1 binding sites. Such promoters therefore appear not to be regulated by methylation. It is possible that the binding of Sp1 to gene promoters with the GGGCGG motif has prevented methylation and the progressive loss of CG dinucleotides and has lead to the development of G+C rich promoters in genes regulated by this transcription factor. As this factor is ubiquitous it follows that these genes will also be ubiquitous; this is indeed the case for house keeping genes.

Although the *periaxin* core promoter is TATA-less it has a very low proportion of CG dinucleotides (0.6 per 100 bp compared to 6-10 for random pairing) which strongly suggests a methylated state and indicates that the tissue-specific and stage-specific expression of the protein is - in part - regulated by a methylation-demethylation mechanism. A similar CG suppression is seen in the promoters of the major myelin genes (Table 3). It is tempting to speculate that activation of a single protein (perhaps by a cAMP dependent kinase or phosphatase) which recognises all of these promoters in their methylated state and/or activation of a specific demethylase could be responsible for the specific upregulation of the transcription of these genes during the Schwann cell myelination programme. Such a mechanism would obviously require the presence of shared features ie common binding sites or DNA conformations. The identification of a number of sequences common to the promoters of many of the myelin genes (as well as periaxin) with as yet undefined properties certainly leaves this possibility open.

4.5 S- and L-Periaxin are differentially Localized Within Myelinating Schwann Cells

An important consequence of the retention of intron 6 within the protein coding sequence of the 5.2 kb periaxin mRNA is that it introduces an in-frame stop codon. Translation of this mRNA therefore results in the generation of a truncated protein which has been named S(small)-periaxin. The previously described 147 kDa periaxin protein encoded by the 4.6 kb mRNA (Gillespie et al, 1994) has also, logically, been renamed as L(large)-periaxin. The function of both of these proteins in myelinating Schwann cells was subsequently addressed.

The distinct subcellular localizations of the two isoforms within myelinating Schwann cells as demonstrated by indirect immunofluorescence appears to suggest a contrasting function for S-periaxin. Whereas L-periaxin is seen to associate with the Schwann cell adaxonal, abaxonal and plasma membranes, S-periaxin is diffuse

TABLE 3

Gene	Species	CG/100 nt	Reference
PMP2	Human	1.2	Hayasaka et al. (1993a)
Po	Human	0.4	Hayasaka et al. (1993b)
	Mouse	0.7	You et al. (1991)
MOG	Mouse	1.0	Daubas et al. (1994)
MAG	Mouse	0.3	Nakano et al. (1991)
	Rat	1.5	Montag, D. (unpublished)
MBP	Human	1.4	Streicher et al. (1989)
	Shark	1.6	Fors et al. (1993)
PLP	Rat	0.3	Boison and Stoffel (1989)
Periaxin	Mouse	0.6	This thesis

The core promoters of most myelin genes are CG-suppressed. Promoter sequences were obtained from Genbank and their dinucleotide content analysed using Macvector sequence analysis software (Oxford Molecular Goup). By random pairing each dinucleotide should have a frequency of approximately 6.25 ie occur 6.25 times in every 100 nt. The myelin gene promoters (plus periaxin) have values between 4 and 30 times lower than would be expected.

throughout the cytoplasm. This discovery was made even more intriguing by the finding that the sequence at the N-terminus of both isoforms shares some similarity with the PDZ domain, a well characterized motif found in a number of proteins (Figure 30). This domain is believed to instigate the recruitment of proteins to the plasma membrane (Kornau et al, 1995). Similarly, PDZ domain-containing proteins may link transmembrane proteins with the actin cytoskeleton via actin-binding proteins (Lue et al, 1994; Marfatia et al, 1996). Given that L-periaxin was initially identified as a potential cytoskeleton-associated protein (Gillespie et al, 1994) and that it has been shown to be cortically targeted, it is exciting to consider the possibility that this domain is responsible for the localization of L-periaxin within Schwann cells. However, the presence of the same domain in S-periaxin appears insufficient to ensure cortical targeting. It is possible that the unique, intron-encoded C-terminus of S-periaxin might target the protein away from the plasma membrane but, equally likely is that there must be other cortical targeting signals present within the L-periaxin.

If the S-periaxin isoform is indeed functional in Schwann cells it is a reasonable assumption that the PDZ domain is involved. It has been suggested that certain PDZ domain containing proteins may play a role in nuclear signalling and transcriptional regulation (Gottardi et al, 1996; Poulat et al, 1997) and it is interesting to note that nuclear S-periaxin staining was occasionally visible. The absence of any known nuclear targetting signal within S-periaxin would suggest that this isoform is not actively transported into the nucleus but proteins of small molecular mass may enter passively (Dingwall and Laskey, 1986), leaving this possibility open. Given that *Prx* is one of the first genes to become transcriptionally active during myelination it is perhaps feasible that as well as a possible role in the myelin structure (the L- isoform) periaxin may also be involved in the transcriptional regulation of the myelin gene program (the S- isoform).

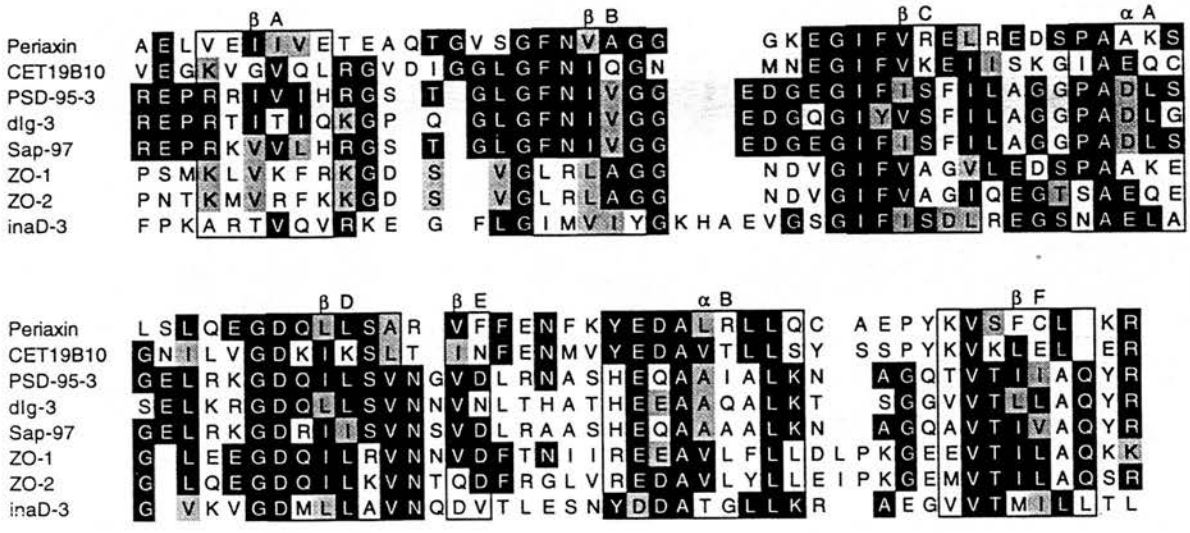


Figure 30. Identification of a PDZ domain at the N-terminus of L- and S-periaxin. The sequence of mouse L- and S-periaxin between amino acids 14 and 98 was compared with PDZ domains in the C. elegans protein CET19B10 (residues 232-316) (Wilson et al, 1994), PSD-95 (residues 309-393) (Cho et al, 1992), Discs-Large (DLG) (residues 482-566) (Woods et al, 1991), SAP-97 (residues 461-545) (Muller et al, 1995), ZO1 (residues 408-491) (Willott et al, 1993), ZO2 (residues 93-176), (Jesaitis et al, 1994) and INAD (residues 361-447) (Shieh et al, 1996). The eight segments within the domain comprise six ß strands and two α-helices (Doyle et al, 1996). Residues shaded black indicate strong conservation within the PDZ motif. Residues in grey indicate a conserved amino acid.

Ongoing from the work of this thesis has been the creation of a knockout mouse by homologous recombination. The *periaxin* gene of these mice lacks most of exon 6 together with all of exon 7 and therefore any periaxin protein which is translated is highly truncated (even more so than S-periaxin). Although these mice form myelin, it is unusually thick (S. Gillespie, personnal communication). Moreover, from about 4 weeks of age peripheral nerve demyelination is clearly visible and is concomitant with a clinical phenotype. This is almost certainly, the clearest indication yet as to the importance of periaxin (either L- or S-) within myelinating Schwann cells.

Future Work

FUTURE WORK

A number of potential protein-protein and protein-DNA interactions have been suggested here to be involved in the functioning of the two periaxin isoforms within myelin-forming Schwann cells. It will be of great interest therefore to see if such interactions can be demonstrated experimentally. Many protein-protein interactions have been successfully identified using the yeast two hybrid system (see Gietz et al, 1997; Fromont-Racine, 1997; Warbrick, 1997 for reviews). To this end, work is currently on-going to identify the functional domains and interacting partners of the L- and S-periaxin isoforms by this method.

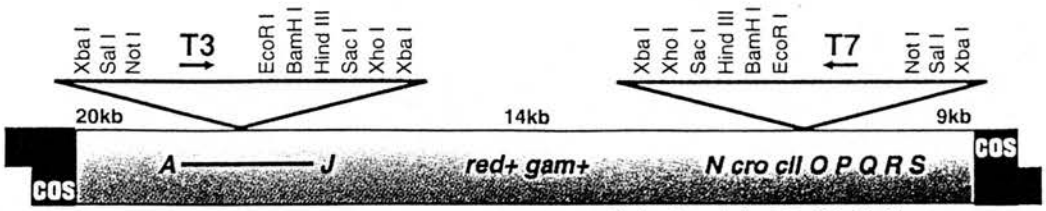
With respect to the transcriptional regulation of the *Prx* gene it will be necessary to identify essential core promoter sequences, enhancers and silencers particularly those responsible for determining Schwann cell expression, for instance by mapping DNase I hypersensitive sites, nuclear extract binding assays and transfection of reporter gene-linked truncated promoter constructs.

As regards the knockout mouse, identifying the molecular events which lead to the mutant phenotype will clearly be vital to our understanding of the role of each isoform during myelination. The reintroduction of each separate isoform into the genome of these mice by transgenics will hopefully yield some definitive answers on the precise nature of the role of both L- and S-periaxin within myelinating Schwann cells.

Appendix

APPENDIX

Map of the Lambda DASH II Replacement Vector



Map of the Lambda DASH® II Vector

Oligonucleotide Primers Specified in Text

- CSG7: 5' GAT GTA CAG CTG TAA GCC GG 3'

Rat periaxin cDNA 122-103 (Gillespie et al, 1994; antisense)

- CSG20: 5' GAT GCA CAG CTG TAA GCA GG 3'

Mouse periaxin cDNA 136-117 (This thesis; antisense)

- LMD30: 5'CGG TGA GTC CTC TCG CAG CTC 3'

Rat periaxin cDNA 426-406 (Gillespie et al, 1994; antisense)

- 5' RACE Anchor: 5' GCC CAC GCG TCG ACT AGT ACG GGG GGG GGG GGG GGG 3'

GIBCO BRL (Sense)

Sequence Comparison of Mouse and Rat 4.6 kb Periaxin

NB. ⇓ indicates splice position within mouse gene.

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                                                    ⇓
M       3 GGAGCTCTGGAGGTGTCTGGAGGCCCACCGAGCCCCAGACCCAGGAGGCC 52
          ||| |||||||||||||||||||||||| |||||||||||||||||||||
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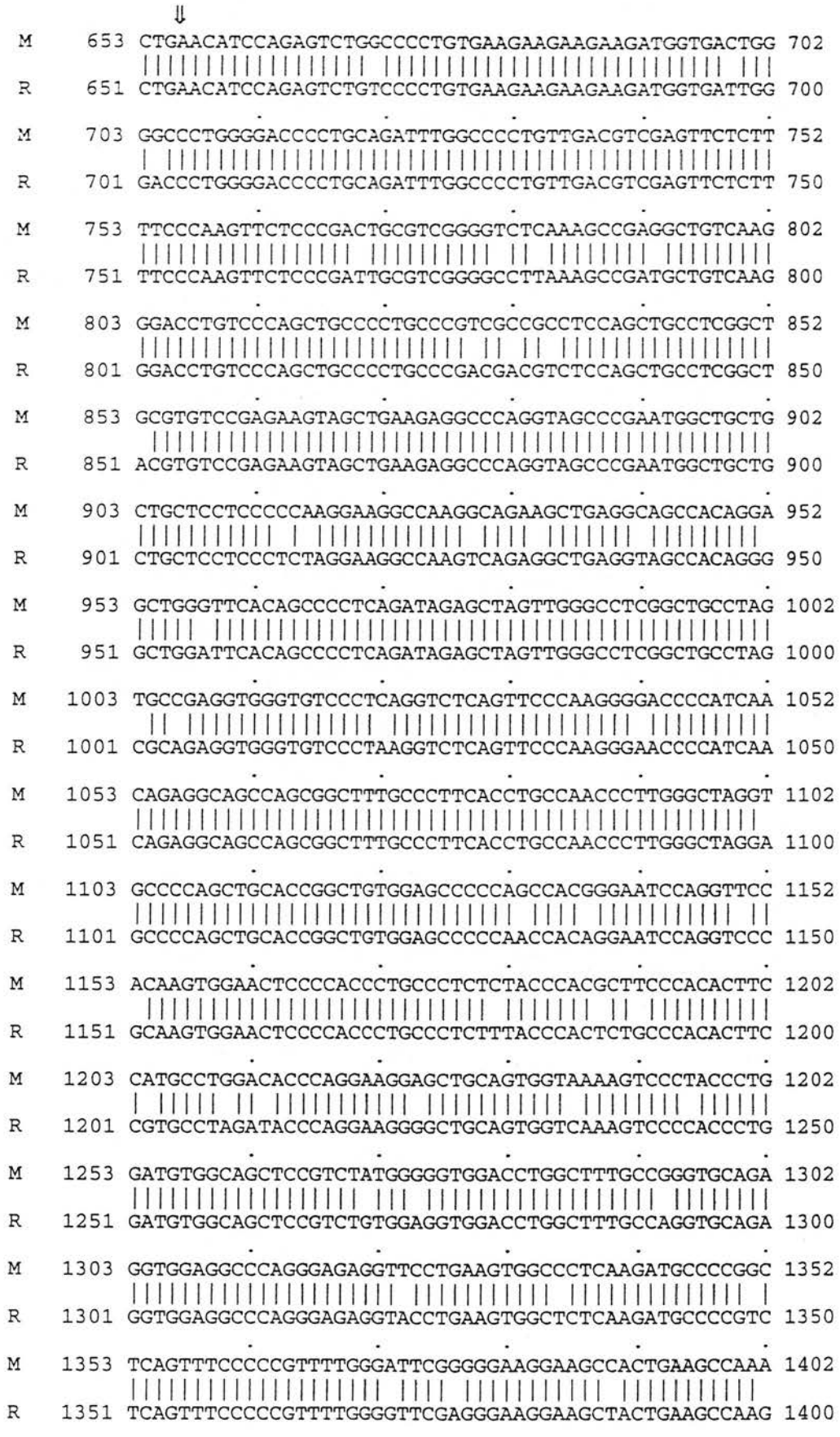
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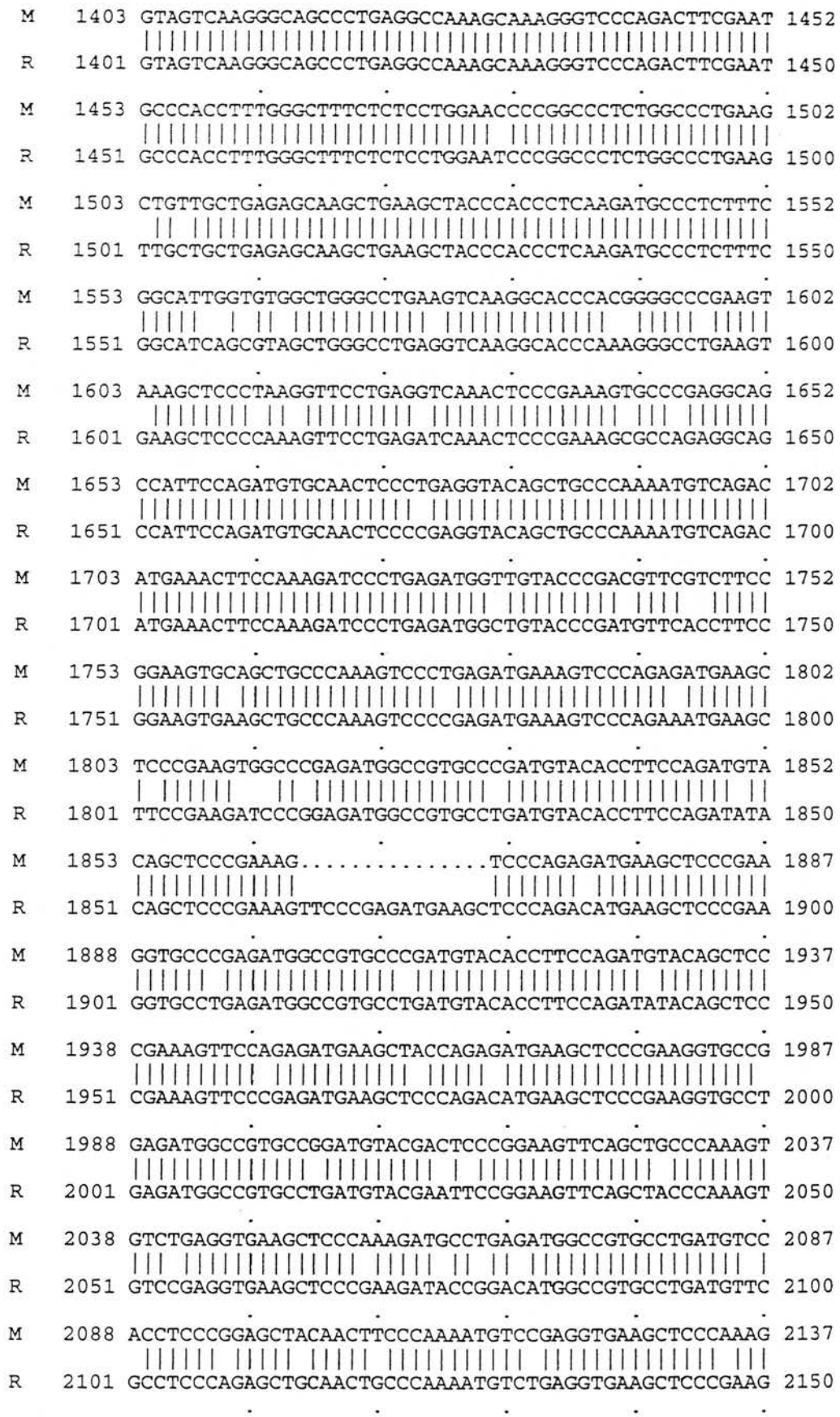

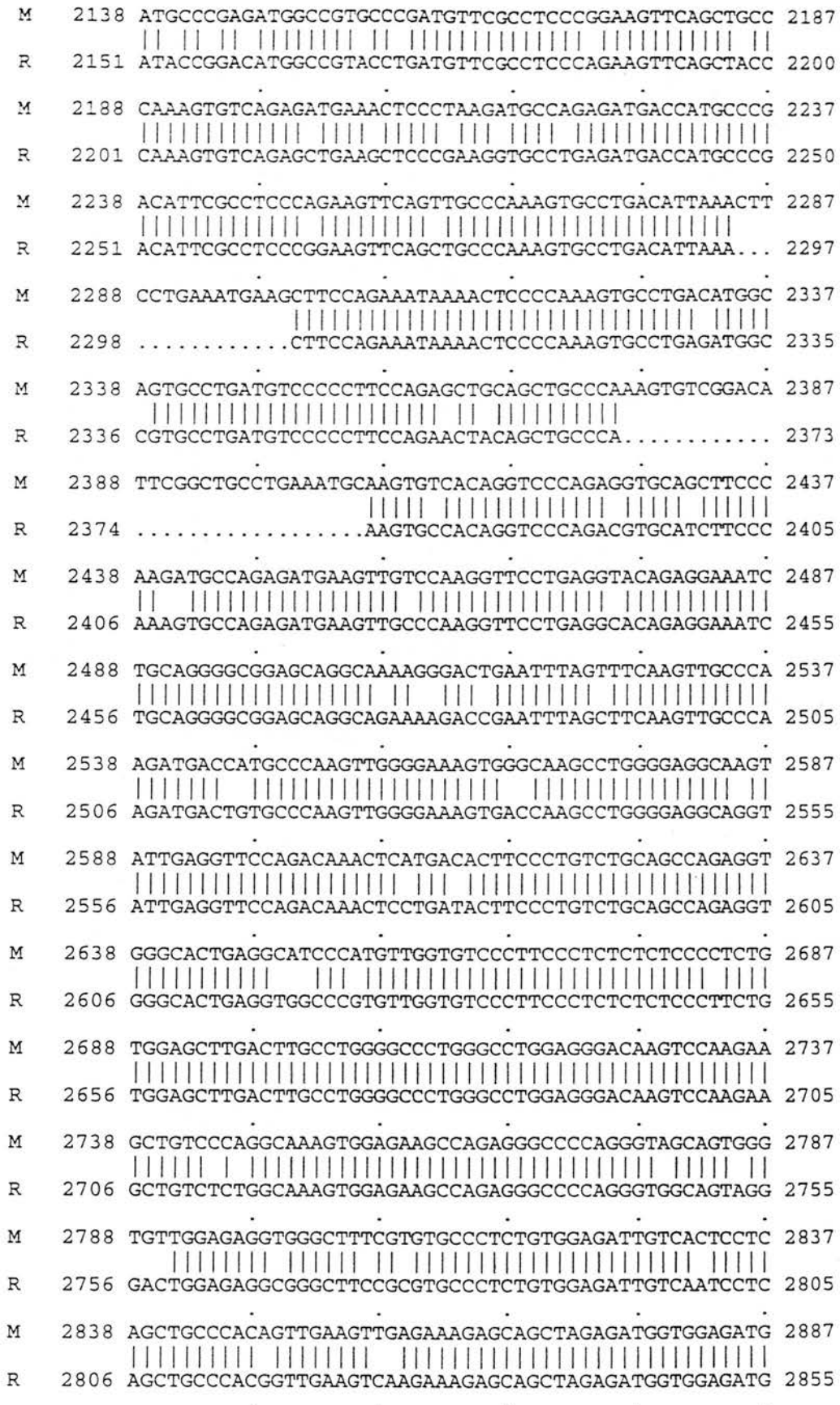
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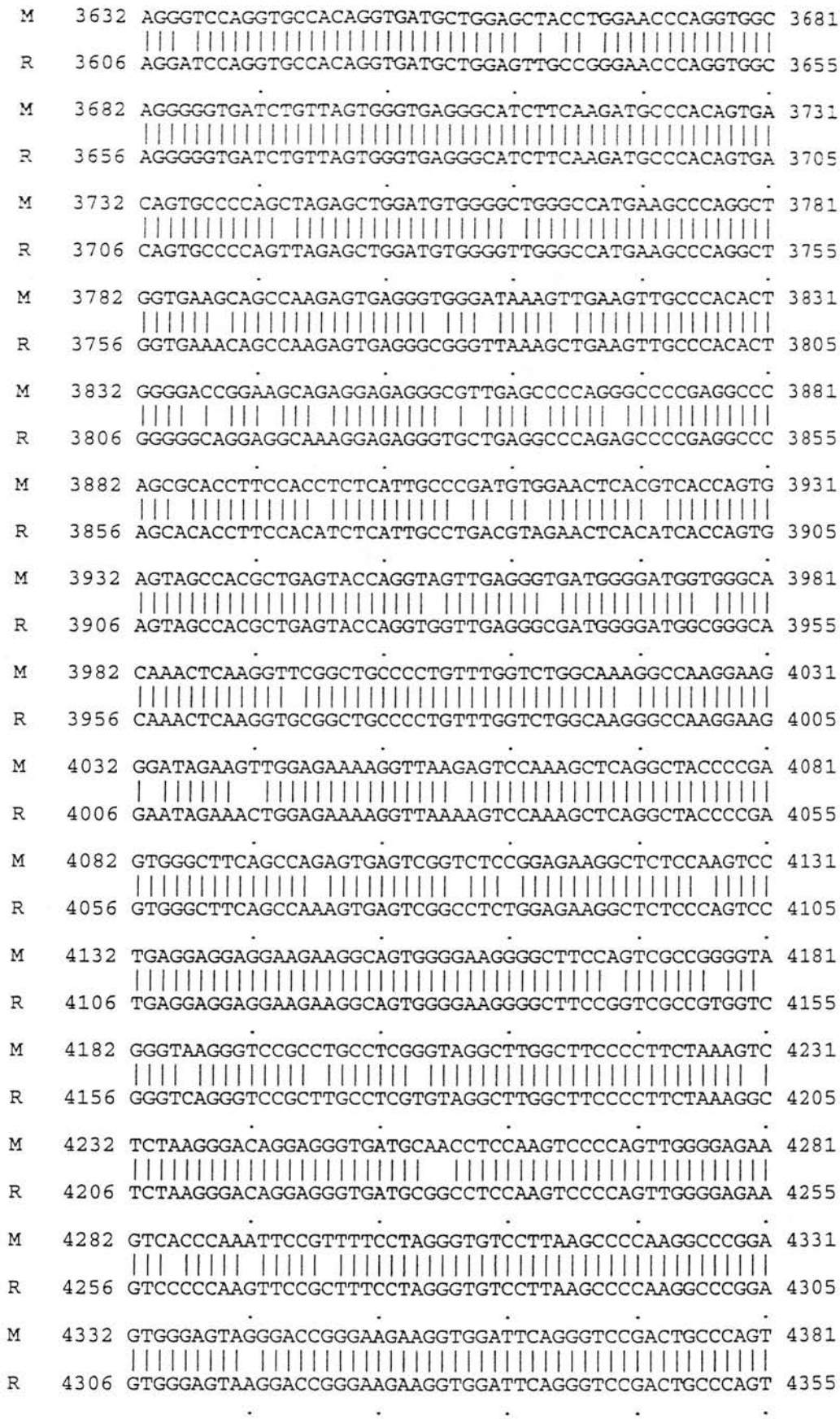
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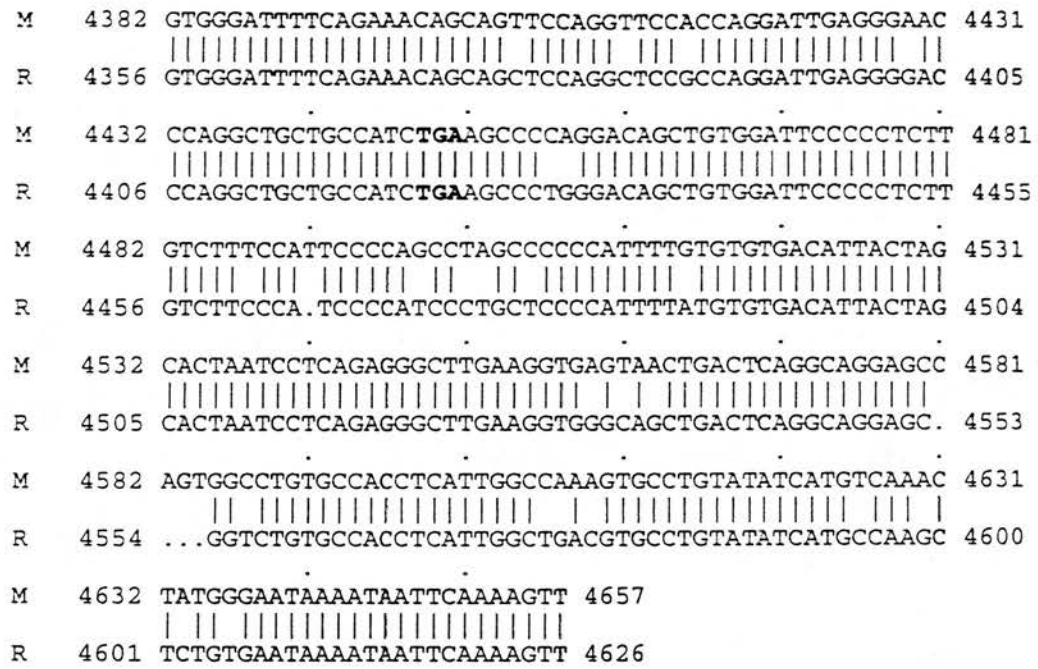
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References

REFERENCES

Adlkofer, K., Frei, R., Neuaberg, D. H., Zielasek, J., Toyka, K. V., and Suter, U. (1997). Heterozygous peripheral myelin protein 22-deficient mice are affected by a progressive demyelinating peripheral neuropathy. J. Neurosci. *17*, 4662-4671.

Adlkofer, K., Martini, R., Aguzzi, A., Zielasek, J., Toyka, K. V., and Suter, U. (1995). Hyper myelination and demyelinating peripheral neuropathy in PMP22-deficient mice. Nat. genet. *11*, 274-280.

Agrawal, H. C., and Agrawal, D. (1991). Proteolipid protein and DM-20 are synthesized by Schwann cells, present in myelin membrane, but they are not fatty acylated. Neurochem. Res. *16*, 855-858.

Agrawal, H. C., Burton, R. M., Fishman, M. A., Mitchell, R. F., and Prensky, A. L. (1972). Partial characterization of a new myelin protein component. J. Neurochem *19*, 2083-2089.

Agrawal, H. C., and Hartman, B. K. (1980). Proteolipid protein and other proteins of myelin. In Proteins of the Nervous System, R. A. Bradshaw and D. M. Schnider, eds. (New York: Raven Press), pp. 145-169.

Agrawal, H. C., Noronha, A. B., Agrawal, D., and Quarles, R. H. (1990). The myelin associated glycoprotein is phosphorylated in the peripheral nervous system. Biochim. Biophys. Res. Commun. *169*, 953-958.

Aguayo, A. J., Attiwell, M., Trecarten, J., Perkins, C. S., and Bray, C. M. (1977). Abnormal myelination in transplanted trembler mouse Schwann cells. Nature *265*, 73-75.

Aguayo, A. J., Chavron, L., and Bray, G. M. (1976). Potential of Schwann cells from unmyelinated nerves to produce myelin: a quantitative ultrastructural and radiographic study. J. Neurocytol. *5*, 565-573.

Aguayo, A. J., Epps, J., Charron, L., and Bray, G. M. (1976). Multipotentiality of Schwann cells in cross-anastomosed and grafted myelinated and unmyelinated nerves: quantitative microscopy and radioautography. Brain Res. 104, 1-20.

Allen, I. V. (1984). Demyelinating diseases. In Greenfield's Neuropathology, J. H. Adams, J. A. N. Corsellis and L. W. Duchen, eds. (London: Edward Arnold), pp. 338-384.

Anderson, D. J. (1993). Cell and molecular biology of neural crest cell lineage diversification. Curr. Opin. Neurobiol. *3*, 8-13.

Anzini, P., Neuberg, D. H., Schachner, M., Nelles, E., Willecke, K., Zielasek, J., Toyka, K., Suter, U., and Martini, R. (1997). Structural abnormalities and deficient maintenance of peripheral nerve myelin in mice lacking the gap junction protein connexin32. J. Neurosci. *17*, 4545-4561.

Arquint, M., Roder, J., Chia, L. S., Down, J., Wilkinson, D., Bayley, H., Braun, P., and Dunn, R. (1987). Molecular cloning and promary structure of myelin-associated glycoprotein. Proc. Natl. Acad. Sci. USA *84*, 600-604.

Aruga, J., Okano, H., and Mikoshiba, K. (1991). Identification of the new isoforms of mouse myelin basic protein: the existence of exon 5a. J. Neurochem. *56*, 1222-1226.

Ashbury, A. K. (1975). The biology of Schwann cells. In Peripheral Neuropathy, P. J. Dyck, P. K. Thomas and E. H. Lambert, eds. (Philadelphia: W.B. Saunders), pp. 201-212.

Barbarash, G. R., Figlewicz, D. A., and Quarles, R. H. (1981). Myelin associated glycoprotein: purification and partial characterization. Trans. Amer. Soc. Neurochem. *12*, 165.

Barbu, M. (1990). Molecular cloning of cDNAs that encode the chicken P0 protein: evidence for early expression in avians. J. Neurosci. Res. *25*, 143-151.

Barclay, A. N., Johnson, P., McCaughan, G. W., and Williams, A. F. (1987). Immunoglobulin-related structures associated with vertebrate cell surfaces. In The T Cell Receptors, T. W. Mak, ed. (New York: Plenum Press).

Ben Othmane, K., Hentati, F., Lennon, F., Ben Hamida, C., Blel, S., Roses, A. D., Pericak-Vance, M. A., Ben Hamida, M., and Vance, J. M. (1993). Linkage of a locus (CMT4A) for autosomal recessive Charcot-Marie-Tooth disease to chromosome 8q. Hum. Mol. Genet. *2*, 1625-1628.

Benga, G., and Holmes, R. P. (1984). Interactions between components in biological membranes and their implications for membrane function. Prog. in Biophys. Molec. Biol. *43*, 195-257.

Benjamins, J. A., and Smith, M. E. (1984). Metabolism of myelin. In Myelin, P. Morell, ed. (New York: Plenum Press), pp. 225-258.

Bergoffen, J., Scherer, S. S., Wang, S., Scott, M. O., Bone, L. J., Paul, D. L., Chen, K., Lensch, M. W., Chance, P. F., and Fischbeck, K. H. (1993). Connexin mutations in X-linked Charcot-Marie-Tooth disease. Science *262*, 2039-2042.

Bermingham, J. R., Scherer, S. S., O'Connell, S., Arroyo, E., Kalla, K. A., Powell, F. L., and Rosenfeld, M. G. (1996). Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration. Genes Dev. *10*, 1751-1762.

Bernier, L., Alvarez, F., Norgard, E. M., Raible, D. W., Mentaberry, A., Schembri, J. G., Sabatini, D. D., and Colman, D. R. (1987). Molecular cloning of a 2',3'-cyclic nucleotide 3'-phosphodiesterase: mRNAs with different 5' ends encode the same set of proteins in nervous and lymphoid tissues. J. Neurosci. *7*, 2703-2710.

Bird, A. P. (1987). CpG islands as gene markers in the vertebrate nucleus. Trends in Genetics *3*, 342-347.

Bird, T. D., Farell, D. F., and Sumo, S. M. (1978). Brain lipid composition of the shiverer mouse: genetic defect in myelin development. J. Neurochem. *31*, 387-391.

Bird, T. D., Ott, J., and Giblett, E. R. (1982). Evidence for linkage of Charcot-Marie-Tooth neuropathy to the duffy locus on chromosome 1. Am. J. Hum. Genet. *34*, 388-394.

Blanchard, A. D., Sinanan, A., Parmantier, E., Zwart, R., Broos, L., Meijer, D., Meier, C., Jessen, K. R., and Mirsky, R. (1996). Oct-6 (Scip/Tst-1) is expressed in Schwann-cell precursors, embryonic Schwann-cells, and postnatal myelinating Schwann-cells: Comparison with Oct-1, Krox-20, and Pax-3. J. Neurosci. Res. *46*, 630-640.

Boison, D., and Stoffel, W. (1989). Myelin deficient rat: a point mutation in exon III (A-C, Thr75-Pro) of the myelin proteolipid protein causes dysmyelination and oligodendrocyte death. Biol. Chem. Hoppe-Seyler *374*, 507-517.

Bone, L. J., Dahl, N., Lensch, M. W., Chance, P. F., Kelly, T., Le Guern, E., Magi, S., Parry, G., Shapiro, H., and Wang, S. (1995). New connexin32 mutations associated with X-linked Charcot-Marie-Tooth disease. Neurology *45*, 1863-1866.

Bosio, A., Binczek, E., and Stoffel, W. (1996). Functional breakdown of the lipid bilayer of the myelin membrane in central and peripheral nervous system by disrupted galactocerebroside synthesis. Proc. Natl. Acad. Sci. USA *93*, 13280-13285.

Braun, P. E. (1984). Molecular organization of myelin. In Myelin, P. Morell, ed. (New York: Plenum Press), pp. 98-113.

Braun, P. E., Bambrick, L. L., Edwards, A. M., and Bernier, L. (1990). 2',3'-cyclic nucleotide 3'-phosphodiesterase has characteristics of cytoskeletal proteins: a hypothesis for its function. Ann. N.Y. Acad. Sci *605*, 55-65.

Braun, P. E., De Angelis, D., Shtybel, W. W., and Bernier, L. (1991). Isoprenoid modification permits 2',3'-cyclic nucleotide 3'-phospodiesterase to bind to membranes. J. neurosci. Res. *30*, 540-544.

Breathnach, R., Benoist, C., O'Hare, K., Gannon, F., and Chambon, P. (1978). Ovalbumin gene: evidence for a leader sequence in mRNA and DNA sequences at the exon-intron boundaries. Proc. Natl. Acad. Sci. USA *75*, 4853-4857.

Breitbart, R. E., Andreadis, A., and Nadal-Ginard, B. (1987). Alternative splicing: a ubiquitous mechanism for the generation of multiple protein isoforms from single genes. Ann. Rev. Biochem. *56*, 467-495.

Brockes, J. P., Raff, M. C., Nishiguchi, D. J., and Winter, J. (1979). Studies on cultured rat Schwann cells III. Assays for peripheral myelin proteins. J. Neurocytol. *9*, 67-77.

Bronner-Fraser, M. (1986). Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal HNK-1. Dev. Biol. *115*, 44-55.

Brophy, P. J. (1992). Interactions of lipids with proteinsof myelin and its associated cytoskeleton. In Myelin: Biology And Chemistry, R. E. Martenson, ed.: CRC Press), pp. 197-212.

Brostoff, S. W., Karkhanis, Y. D., Carlo, D. J., Reuter, W., and Eylar, E. H. (1975). Isolation and partial characterization of the major proetins of rabbit sciatic nerve myelin. Brain Res. *86*, 449-458.

Bunge, M. B., Bunge, R. P., and Pappas, G. D. (1962). Electron microscopic demonstrations of connections between glia and myelin sheaths in the developing mammalian central nervous system. J. Cell Biol. *12*, 448.

Bunge, M. B., Williams, A. K., and Wood, P. M. (1982). Neuron-Schwann cell interaction in basal lamina formation. Dev. Biol. *92*, 449-460.

Bunge, R. P., Bunge, M. B., and Eldridge, C. F. (1986). Linkage between axonal ensheathment and basal lamina production by Schwann cells. Annu. Rev. Neurosci. *9*, 305-328.

Bunge, R. P., Bunge, M. B., and Eldridge, C. F. (1989). Movements of the Schwann cell nucleus implicate progression of the inner (axon-related) Schwann cell process during myelination. J. Cell Biol. *109*, 273-284.

Busslinger, M., Hurst, J., and Flavell, R. A. (1983). DNA methylation and the regulation of globin gene expression. Cell *34*, 197-206.

Campagnoni, A. T. (1995). Molecular Biology of Myelination. In Neuroglia, H. Kettenmann and B. R. Ransom, eds.: Oxford University Press), pp. 555-570.

Campagnoni, A. T., Pribyl, T. M., Campagnoni, C. W., Kampf, K., Amur-UmarJee, S., Landry, C. F., Handley, V. W., Newman, S. L., Garbay, B., and Kitamura, K. (1993). Structure and developmental expression of Golli-mbp, a 105-kilobase gene that encompasses the myelin basic protein gene and is expressed in cells in the oligodendrocyte lineage in the brain. J. Biol. Chem. *286*, 4930-4938.

Chabre, M. (1975). X-ray diffraction studies of retinal rods 1. Structure of the disc membrane, effect of illumination. Biochim. Biophys. Acta *382*, 322.

Chance, P. F., Alderson, M. K., Lepping, K. A., Lensch, M. W., Matsunami, N., Smith, B., Swanson, P. D., Odelberg, S. J., Disteche, C. M., and Bird, T. D. (1993). DNA deletion associated with hereditary neuropathy with liability to pressure palsies. Cell *72*, 143-151

Chance, P. F., Bird, T. F., Matsunami, N., Lensch, M. W., Brothman, A. R., and Feldman, G. M. (1992). Trisomy 17p associated with Charcot-Marie-Tooth neuropathy type 1a phenotype. Neurology *42*, 2295-2299.

Cho, K. O., Hunt, C. A., and Kennedy, M. B. (1992). The rat brain post-synaptic density fraction contains a homolog of the Drosophila Discs-Large tumor suppressor protein. Neuron *9*, 929-942.

Coetzee, T., Fujita, N., Dupree, J., Shi, R., Blight, A., Suzuki, K., and Popko, B. (1996). Myelination in the absence of galactocerebroside and sulfatide: normal structure with abnormal function and regional instability. Cell *86*, 209-219.

Corden, J., Wasylyk, B., Buckwalder, A., Sassone-Corsi, P., Kedinger, C., and Chambon, P. (1980). Promoter sequences of eukaryotic protein-coding genes. Science *209*, 1406-1414.

Crabb, J. W., and Saari, J. C. (1981). N-terminal sequence homology among retinoid-binding proteins from bovine retina. FEBS letts. *130*, 15-18.

D'Urso, D., Brophy, P. J., Staugaitus, S. M., Gillespie, C. S., Frey, A. B., Stempak, J. G., and Colman, D. R. (1990). Protein zero of peripheral nerve myelin: biosynthesis, membrane insertion and evidence for homotypic interaction. Neuron *2*, 449-460.

Daemon, F. J. M. (1973). Vertebrate rod outer segment membranes. Biochim. Biophys. Acta. *300*, 255.

Dahm, L. M., and Landmesser, L. (1988). The regulation of intramuscular nerve branching during normal development and following activity blockade. Dev. Biol. *130*, 621.

Darnell, J. E., and Doolittle, W. F. (1986). Speculations on the early course of evolution. Proc. Natl. Acad. Sci. USA *83*, 1271-1275.

Daubas, P., Phamdinh, D., and Dautigny, A. (1994). Structure and polymorphism of the mouse myelin/oligodendrocyte glycoprotein gene. Genomics *23*, 36-41.

de Ferra, F., Engh, H., Hudson, L., Kamholz, J., Puckett, C., Molineaux, S., and Lazzarini, R. A. (1985). Alternative splicing accounts for the four forms of myelin basic protein. Cell *43*, 721-727.

De Vries, G. H., Salzer, J. L., and Bunge, R. P. (1982). Axolemma-enriched fractions isolated from PNS and CNS are mitogenic for Schwann cells. Dev. Brain Res. *3*, 295-299.

Deibler, G. E., Stome, A. L., and Kies, M. W. (1990). Role of phosphorylation in conformational adaptability of bovine myelin basic protein. Proteins *7*, 32-40.

Devereaux, J. P., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. *12*, 387-395.

Dieperink, M. E., O'Neill, A., Magnoni, G., Wellman, R. L., Helnrikson, R. L., Zurcher-Neely, H. A., and Stefansson, K. (1992). SAG: a Schwann cell membrane glycoprotein. J. Neurosci. 12 *2177-2185.*

Ding, Y., and Brunden, K. R. (1994). The cytoplasmic domain of myelin protein P0 interacts with negatively charged phospholipid bilayers. J. Biol. Chem. *269*, 10764-10770.

Dingwall, C., and Laskey, R. A. (1986). Protein import into the cell nucleus. Annu. Rev. Cell Biol. *2*, 367-390.

Dirksen, W. P., Sun, Q., and Rottman, F. M. (1995). Multiple splicing signals control alternative intron retention of Bovine growth hormone pre-mRNA. J. Biol. Chem. *270*, 5346-5352.

Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R., and Jessen, K. R. (1995). Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. Neuron. 15, 585-596

Doyle, D. A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R. (1996). Crystal structures of a complexed and peptide-free membrane protein-binding domain: Molecular basis of peptide recognition by PDZ. Cell *85*, 1067-1076.

Dyck, P. J., and Thomas, P. K. (1995). Peripheral Neuropathology (Philadelphia: W.B. Saunders).

Edelman, G. M. (1983). Cell adhesion molecules. Science *219*, 450-457.

Edwards, A. M., Arquint, M., Braun, P. E., Roder, J. C., Dunn, R., Pawson, T., and Bell, J. C. (1988). Myelin associated glycoprotein, a cell adhesion molecule of oligodendrocytes, is phosphorylated in brain. Mol. Cell Biol. *8*, 2655-2658.

Eichberg, J., and Iyer, S. (1996). Phosphorylation of myelin proteins: recent advances. Neurochem. Res. *21*, 527-535.

Eriksson, U., Sundelin, J., Rask, L., and Peterson, P. A. (1981). The NH2 terminal amino acid sequence of cellular retinoic acid binding protein from rat testis. FEBS Lett. *135*, 70-72.

Every, J. L., Brady, R. O., and Quarles, R. H. (1973). Evidence that the major protein of rat sciatic nerve myelin is a glycoprotein. J. Neurochem. *21*, 329.

Fairweather, N., Bell, C., Cochrane, S., Chelly, J., Wang, S., Mostacciuolo, M. L., Monaco, A. P., and Haites, N. E. (1994). Mutations in the connexin 32 gene in X-linked dominant Charcot-Marie-Tooth disease (CMTX1). Hum. Mol. Genet. *3*, 29-34.

Faisst, S., and Meyer, S. (1992). Compilation of vertebrate-encoded transcription factors. Nucl. Acids Res. *20*, 3-26.

Falconer, D. S. (1951). Two new mutations, trembler and reeler, with neurological action in the house mouse. J. Genet. *50*, 192-201.

Fanning, A. S., and Anderson, J. M. (1996). Protein-protein interactions - PDZ domain networks. Curr. Biol. *6*, 1385-1388.

Figlewicz, D. A., Quarles, R. H., Johnson, D., Barbarash, G. R., and Sternberger, N. H. (1981). Biochemical demonstration of the myelin asssociated glycoprotein in the peripheral nervous system. J. Neurochem *27*, 749-758.

Filbin, M. T., and Tennekoon, G. I. (1991). The role of complex carbohydrates in adhesion of the myelin protein, P0. Neuron *7*, 845-855.

Filbin, M. T., Walsh, F. S., Trapp, B. D., Pizzey, J. A., and Tennekoon, G. I. (1990). Role of myelin P0 protein as a homophilic adhesion molecule. Nature *344*, 871-872.

Finean, J. B., Colman, R., and Michell, R. H. (1984). Membranes and Their Cellular Functions: Blackwell scientific Publications).

Fink, G. R. (1987). Pseudogenes in yeast? Cell *49*, 5-6.

Folch-Pi, J., and Lees, M. (1951). Proteolipid - a new type of tissue lipoproteins. J. Biol. Chem. *191*, 807-817.

Fong, J. W., Ledeen, R. W., Kundo, S. K., and Brostoff, S. W. (1976). Gangliosides of peripheral nerve myelin. J. Neurochem. *26*, 157-162.

Fors, L. (1993). Sequence similarities of myelin basic protein promoters from mouse and shark - implications for the control of gene expression in myelinating cells. J. Neurochem. *60*, 1585-1585.

Frail, D. E., Webster, H. D., and Braun, P. E. (1985). Developmental expression of the myelin associated glycoprotein in the peripheral nervous system is different from that in the central nervous system. J. Neurochem. *45*, 1308-1310.

Frank, E., and Sanes, J. R. (1991). Lineage of neurons and glia in chick dorsal root ganglia: analysis in vivo with a recombinant retrovirus. Development *111*, 895-908.

Fraser, S. E., and Bronner-Fraser, M. (1991). Migrating neural crest cells in the trunk of the avian embryo are multipotent. Development *112*, 913-920.

Friede, R. L. (1972). Control of myelin formation by axon calibre (with a model of the control mechanism). J. Comp. Neurol. *144*, 233-236.

Friede, R. L., and Bischhausen, R. (1982). How are sheath dimensions affected by axon caliber and internode length? Brain Res. *235*, 335-350.

Friede, R. L., and Samorajski, T. (1967). Relation between the number of myelin lamellae and axon circumference in fibres of vagus and sciatic nerves in mice. J. Comp. Neurol. *130*, 223-231.

Fromont-Racine, M., Rain, J. C., and Legrain, P. (1997). Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens. Nature Genet. *16* , 277-282.

Fruttiger, M., Montag, D., Schachner, M., and Martini, R. (1995). Crucial role for the myelin associated glycoprotein in the maintenance of axon-myelin assembly. Eur. J. Neurosci. *7*, 511-515.

Fujita, N., Sato, S., Kurihara, T., Kuwano, R., Sakimura, K., Inuzuka, T., Takahashi, Y., and Miyatake, T. (1989). cDNA cloning of mouse myelin-associated glycoprotein: a novel alternative splicing pattern. Biochim. Biophys. Res. Commun. *165*, 1162-1169.

Gabreels-Festern, A., Bolhuis, P. A., Hoogendijk, J. E., Valentijn, L. J., Eshuis, E., and Gabreels, F. J. M. (1995). Charcot-Marie-Tooth type 1a: morphological phenotype of the 17p duplication versus PMP22 point mutations. Acta. Neuropathol. *90*, 645-649.

Gamble, H. J. (1966). Further electron microscope studies on human foetal peripheral nerves. J. Anat. *100*, 487-498.

Gamble, H. J., and Breathnach, A. S. (1965). An electron microscope study of human foetal peripheral nerves. J. Anat. *99*, 573-584.

Ganser, A. L., and Kirschner, P. A. (1980). Myelin structure in the absence of basic protein in the Shiverer mouse. In Neurological Mutations Affecting Myelination, N. Baumann, ed. (Amsterdam: Elsevier), pp. 171-176.

Garbern, J. Y., Cambi, F., Tang, X.-M., Sima, A. A. F., Vallat, J. M., Bosch, E. P., Lewis, R., Shy, M., Sohi, J., Kraft, G., Chen, K. L., Joshi, I., Leonard, D. G. B., Johnson, W., Raskind, W., Dlouhy, S. R., Pratt, V., Hodes, M. E., Bird, T., and Kamholz, J. (1997). Proteolipid protein is necessary in peripheral as well as central myelin. Neuron *19*, 205-218.

Gavrilovic, J., Brennan, A., Mirsky, R., and Jessen, K. R. (1995). Fibroblast growth factors and insulin growth factors combine to promote survival of rat Schwann cell precursors without induction of DNA synthesis. Eur. J. Neurosci. *7*, 77-85.

Geren, B. B. (1954). The formation from the Schwann cell surface of myelin in peripheral nerves of chick embryos. Exp. Cell. Res. *7*, 558-566.

Giese, K. P., Martini, R., Lemke, G., Soriano, P., and Schachner, M. (1992). Mouse P0 gene disruption leads to hypomyelination, abnormal expression of recognition molecules and degeneration of myelin and axons. Cell *71*, 565-576.

Gietz, R. D., Raine, B., Robbins, A., Graham, K. C., and Woods, R. A. (1997). Identifiactions of proteins that interact with a protein of interest: applications of the yeast two hybrid system. Mol. Cell Biochem. *172*, 67-79.

Gilbert, W. (1987). The exon theory of genes. CSH Symp. Quant. Biol. *LII*, 901-905.

Gilbert, W. (1978). Why genes in pieces? Nature *271*, 501.

Gillespie, C. S., Lee, M., Fantes, J. F., and Brophy, P. J. (1997). The gene encoding the Schwann cell protein periaxin localizes on mouse chromosome 7(Prx). Genomics *41*, 297-298.

Gillespie, C. S., Sherman, D. L., Blair, G. E., and Brophy, P. J. (1994). Periaxin, a novel protein of myelinating Schwann cells with a possible role in axonal ensheathment. Neuron *12*, 497-508.

Gottardi, C. J., Arpin, M., Fanning, A. S., and Louvard, D. (1996). The junction-associated protein, zonula occludens-1. localizes to the nucleus before the maturation and during the remodeling of cell-cell contacts. Proc. Natl. Acad. Sci. USA *93*, 10779-10784.

Gould, R. M. (1992). The cell of Schwann: an update. In Myelin: Biology and Chemistry, R. E. Martenson, ed.: CRC Press).

Gravel, M., DeAngelis, D., and Braun, P. E. (1994). Molecular cloning and characterisation of rat brain 2',3'-cyclic nucleotide 3'-phosphodiesterase isoform 2. J. Neurosci. Res. *38*, 243-247.

Greenfield, S., Brostoff, S., Eylar, E. H., and Morell, P. (1973). Protein composition of myelin of the peripheral nervous system. J. Neurochem. *20*, 1207-1216.

Greenfield, S., Brostoff, S. W., and Hogan, E. L. (1980). Charcterisation of the basic proteins from rodent peripheral nervous system myelin. J. Neurochem. *34*, 453-455.

Greenfield, S., Weise, M. J., Gantt, G., Hogan, E. L., and Brostoff, S. A. (1982). Basic proteins of rodent peripheral nerve myelin: immunochemical identification of the 21.5 kd, 18.5 kd, 17 kd, 14 kd, and P2 protein. J. Neurochem *39*, 1278-1283.

Griffiths, I. R. (1996). Myelin mutants: model systems for the study of normal and abnormal myelination. Bioessays *18*, 789-797.

Hanemann, C. O., Stoll, G., D'Urso, D., Fricke, W., Martin, J. J., Van Broeckhoven, C., Mancardi, G. L., Bartke, I., and Muller, H. W. (1994). Peripheral myelin protein-22 expression in Charcot-Marie-Tooth disease type 1a sural nerve biopsies. J. Neurosci. Res. *37*, 654-659.

Hawkins, J. D. (1988). A survey on intron and exon lengths. Nucl. Acids Res. *16*, 9893-9908.

Hayasaka, H., Himoro, M., Takada, G., Takahashi, E., Minoshima, S., and Shimizu, N. (1993). Structure and localization of the gene encoding human peripheral myelin protein 2. Genomics *18*, 244-248.

Hayasaka, K., Himoro, M., Sato, W., Takada, G., Uyemura, K., Shimizu, N., Bird, T. D., Conneally, P. M., and Chance, P. F. (1993). Charcot-Marie-Tooth neuropathy type 1B is associated with muations of myelin P0 gene. Nature Genet. *5*, 31-34.

Hayasaka, K., Himoro, M., Wang, Y. M., Takata, M., Minoshima, S., Shimizu, N., Miura, M., Uyemura, K., and Takada, G. (1993). Structure and chromosomal localization of the gene encoding the human myelin protein zero (MPZ). Genomics *17*, 755-758.

Hayasaka, K., Nanao, K., Tahara, M., Sato, W., Takada, G., Miura, M., and Uyemura, K. (1991). Isolation and sequence determination of cDNA encoding the major structural protein of human peripheral myelin. Biochem. Biophys. Res. Commun. *180*, 515-518.

Henry, E. W., and Sidman, R. L. (1988). Long lives for homozygous trembler mutant mice despite virtual absence of peripheral nerve myelin. Science *241*, 344-346.

Hilmi, S., Fournier, M., Valeins, H., Gandar, J. C., and Bonnet, J. (1995). Myelin P0 glycoprotein: identification of the site phosphorylated in vitro and in vivo by endogenous protein kinases. J. Neurochem. *64*, 902-907.

Hirano, A. (1982). The permeability of the extracellular spaces at the Schmidt-Lanterman clefts and paranodes in peripheral myelin sheaths. Acta Neuropathol. *58*, 34-38.

Hooft van Huijsduijnen, R. A. M., Bollekens, J., Dorn, A., Benoist, C., and Mathis, D. (1987). Properties of a CCAAT box-binding protein. Nucl. Acids Res. *15*, 7265-7282.

Hoogendijk, J. E., Hensels, G. W., Zorn, I., Valentijn, L., Janssen, E. A., de Visser, M., Barker, D. F., Ongerboer de Visser, B. W., Baas, F., and Bolhuis, P. A. (1991). The duplication in Charcot-Marie-Tooth disease type 1a spans at least 1100 kb on chromosome 17p11.2. Hum. Genet. *88*, 215-218.

Horvath, C. M., Wen, Z., and Darnell, J. E. (1995). A STAT protein domain that determines DNA sequence recognition suggets a novel DNA-binding domain. Genes and Dev. *9*, 984-994.

Hudson, L. D., Berndt, J. A., Puckett, C., Kozak, C. A., and Lazzarini, R. A. (1987). Aberrant splicing of proteolipid protein mRNA in the dysmyelinating jimpy muatnt mouse. Proc. Natl. Acad. Sci. USA *84*, 1454-1458.

Ionasescu, V., Searby, C., Ionasescu, R., and Meschino, W. (1995). New point mutations and deletions of the connexin 32 gene in X-linked Charcot-Marie-Tooth neuropathy. Neuromusc. Disorders. *5*, 297-300.

Ishaque, A., Hofmann, T., and Eylar, E. H. (1982). The complete amino acid sequence of the rabbit P2 protein. J. Biol. Chem. *257*, 592-595.

Ishaque, A., Roomi, M. W., Szymanska, I., Kowalski, S., and Eylar, E. (1980). The P0 glycoprotein of peripheral nerve myelin. Can. J. Biochem. *58*, 913-921.

Itoh, M., Nagafuchi, A., Yonemura, S., Kitani-Yasuda, T., Tsukita, S., and Tsukita, S. (1993). The 220 kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and electron microscopy. J. Cell Biol. *121*, 491-502.

Jaegle, M., Mandemakers, W., Broos, L., Zwart, R., Karis, A., Visser, P., Grosveld, F., and Meijer, D. (1996). The POU factor Oct-6 and Schwann cell differentiation. Nature *273*, 507-510.

Jesaitis, L. A., and Goodenough, D. A. (1994). Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the *Drosophila* Discs-Large tumor suppressor protein. J. Cell Biol. *124*, 946-961.

Jessen, K. R., Brennan, A., Morgan, L., Mirsky, R., Kent, A., Hashimoto, Y., and Gavrilovic, J. (1994). The Schwann cell precursor and its fate: a study of cell death and differentiation during gliogenesis in rat embryonic nerves. Neuron *12*, 509-527.

Jessen, K. R., Mirsky, R., and Morgan, L. (1987). Axonal signals regulate the differentiation of non-myelin-forming Schwann cells: an immunohistochemical study of galactocerebroside in transected and regenerating nerves. J. Neurosci. *7*, 3362-3369.

Jessen, K. R., Morgan, L., Brammer, M., and Mirsky, R. (1985). Galactocerebroside is expressed by non-myelin-forming Schwann cells in situ. J. Cell Biol. *101*, 1135-1143.

Jessen, K. R., Morgan, L., Stewart, H. L. S., and Mirsky, R. (1990). Three markers of adult non-myelin-forming Schwann cells, 217c (Ran-1), A5E3 and GFAP: development and regulation by neuron-Schwann cell interactions. Development *109*, 91-103.

Jessen, K. R., and Mirsky, R. (1991). Schwann cell precursors and their development. Glia *4*, 185-194.

Kim, J. B., Spotts, G. D., Halvorsen, Y.-D., Shih, H.-M., Ellenberger, T., Towle, H. C., and Spiegelman, B. C. (1995). Dual DNA binding specificity of ADD1/SREBP1 controlled by a single amino acid in the basic helix-loop-helix domain. Mol. Cell. Biol. *15*, 2582-2588.

Kimura, M., Inoko, H., Katsuki, M., Ando, A., Sato, T., Hirose, T., Takashimi, H., Inayama, S., Okano, H., Takamtsu, K., Mikoshiba, K., Tsukada, Y., and Wanatabe, I. (1985). Molecular genetic analysis of myelin-deficient mice: shiverer mutant mice show deletion in gene(s) coding for myelin basic protein. J. Neurochem. *44*, 692-696.

Kimura, M., Sato, M., Akatsuka, A., Nozawa-Kimura, S., Takahashi, R., Yokoyama, M., Nomura, T., and Katsuki, M. (1989). Restoration of myelin formation by a single type of myelin basic protein in transgenic shiverer mice. Proc. Natl. Acad. Sci. USA *86*, 5661-5665.

Kirschner, D. A., and Ganser, A. L. (1982). Myelin labelled with mercuric chloride:Asymmetric localization of phosphatidylethanolamine plasmalogen. J. Mol. Biol. *157*, 635-658.

Kirschner, D. A., Ganser, A. L., and Caspar, D. L. D. (1984). Diffraction studies of molecular organisation and membrane interactions in myelin. In Myelin, P. Morell, ed. (New York: Plenum Press), pgs 30-74.

Kishimoto, Y. (1983). Ceramides and Cerebrosides. In Handbook of Neurochemistry, A. Lajtha, ed. (New York: Plenum Press), pgs 56-64.

Kitamura, K., Newman, S. L., Campagnoni, C. W., Verdi, J. M., Mohandas, T., Handley, V. W., and Campagnoni, A. T. (1990). Expression of a novel transcript of the myelin basic protein gene. J. Neurochem. *54*, 2032-2041.

Kitamura, K., Suzuki, M., Suzuki, A., and Uyemura, K. (1980). The complete amino acid sequence of the P2 protein in bovine peripheral nerve myelin. FEBS Lett. *115*, 27-30.

Kitamura, K., Suzuki, M., and Uyemura, K. (1976). Purification and partial characterizationof two glycoproteins in bovine peripheral nerve myelin membrane. Biochim. Biophys. Acta. *455*, 806-816.

Koizumi, T., Kimura, M., and Hayakawa, J. (1991). Localization of the gene encoding myelin basic protein to mouse chromosome 18E3-4 and rat chromosome 1p11-p12. Cytogenet. Cell Genet. *56*, 199-201.

Konat, G. W. (1996). Chromatin structure and transcriptional activity of MAG gene. Acta Neurobiol. Exp. *56*, 281-285.

Kornau, H. C., Schenker, L. T., Kennedy, M. B., and Seeburg, P. H. (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science *269*, 1737-1740.

Kroczek, R. A., and Siebert, E. (1990). Optimization of northern analysis by vacuum-blotting, RNA: transfer visualisation and ultraviolet fixation. Analytical Biochemistry *184*, 90-95.

Kruse, F., Mailhammer, R., Wernecke, H., Faissner, A., and Sommer, I. (1984). Neural cell adhesion molecules and myelin associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK-1. Nature *311*, 153-155.

Kuhn, R., Monuki, E. S., and Lemke, G. (1991). The gene encoding the transcription factor SCIP has features of an expressed retroposon. Mol. Cell. Biol. *11*, 4642-4650.

Kuhn, R., Pravtcheva, D., Ruddle, F., and Lemke, G. (1990). The gene encoding peripheral myelin protein zero is located on mouse chromosome 1. J. Neurosci. *10*, 205-209.

Kulkens, T., Bolhuis, P. A., Wolterman, R. A., Kemp, S., te Nijenhuis, S., Valentijn, L. J., Hensels, G. W., Jennekens, F. G. I., de Visser, M., Hoogendijk, J. E., and Baas, F. (1993). Deletion of the serine 34 codon from the major peripheral myelin protein P0 gene in Charcot-Marie-Tooth disease type 1B. Nat. genet. *5*, 35-39.

Kunzelmann, P., Blumcke, I., Traub, O., Dermietzel, R., and Willecke, K. (1997). Coexpression of connexin 45 and -32 in oligodendrocytes of art brain. J. Neurocytol. *26*, 17-22.

Kurihara, T., Nishizawa, Y., Takahashi, Y., and Odani, S. (1981). Chemical, immunological and catalytic properties of 2'3'-cyclic nucleotide 3'-phophodiesterase purified from brain white matter. Biochem. J. *195*, 153-157.

Kurihara, T., and Tsukada, Y. (1967). The regional and subcellular distribution of 2',3'-cyclic nucleotide 3'-phosphodiesterase in the central nervous system. J. Neurochem. *14*, 1167-1174.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T. Nature *227*, 680-685.

Lai, C., Brow, M. A., Nave, K. A., Noronha, A. B., Quarles, R. H., Bloom, F. E., Milner, R. J., and Sutcliffe, J. G. (1987). Two forms of 1B236/myelin-associated glycoprotein, a cell adhesion molecule for post-natal neural development, are produced by alternative splicing. Proc. Natl. Acad. Sci. USA *84*, 4337-4341.

Lamperth, L., Manuelidis, L., and Webster, H. d. F. (1990). P0 glycoprotein: messenger RNA distribution in myeling forming Schwann cells at the developing rat trigeminal ganglion. J. Neurocytol. *19*, 756-769.

Landry, C. F., Ellison, J. A., Pribyl, T. M., Campagnoni, C., Kampf, K., and Campagnoni, A. T. (1996). Myelin basic protein gene expression in neurons: developmental and regional changes in protein targeting within neuronal nuclei, cell bodies and processes. J. Neurosci. *16*, 2452-2462.

Langley, J. N., and Anderson, H. K. (1903). On the union of the fifth cervical nerve with the superior cervical ganglion. J. Physiol. *30*, 439-442.

Le Douarin, N. (1986). Cell line segregation during peripheral nervous system ontogeny. Science *231*, 1515-1522.

Le Douarin, N. (1982). The neural crest (Cambridge: Cambridge University Press).

Le Douarin, N., Dulac, C., Dupin, E., and Cameron-Curry, P. (1991). Glial cell lineages in the neural crest. Glia *4*, 175-184.

Lebo, R. V., Chance, P. F., Dyck, P. J., Redila-Flores, M. T., Lynch, E. D., M.S., G., Bird, T. D., King, M. C., Anderson, L. A., and Hall, J. (1991). Chromosome 1 Charcot-Marie-Tooth disease (CMT1B) locus in the Fc gamma receptor gene region. Hum. Genet. *88*, 1-12.

Lees, M. B., and Brostoff, S. W. (1984). Proteins of Myelin. In Myelin, P. Morell, ed. (New York: Plenum Press), pp. 197-224.

Lemke, G. (1993). The molecular genetics of myelination: an update. Glia *7*, 263-271.

Lemke, G., and Axel, R. (1985). Isolation and sequence of a cDNA encoding the major structural protein of peripheral myelin. Cell *40*, 501-508.

Lemke, G., and Chao, M. (1988). Axons regulate Schwan cell expression of the major myelin and NGF receptor genes. Development *102*, 499-504.

Lemke, G., Lamar, E., and Patterson, J. (1988). Isolation and analysis of the gene encoding peripheral myelin protein zero. Neuron *1*, 73-83.

Li, C., Tropak, M. B., Gerial, R., Clapoff, S., Abramow-Newerly, W., Trapp, B., Peterson, A., and Roder, J. (1994). Myelination in the absence of myelin-associated glycoprotein. Nature *369*, 747-750.

Li, J. (1978). An X-ray diffraction study of chloroplast thylakoid membrane structure. In Harvard University (Cambridge, Massachusetts.

Li, X., Wrabetz, L., Cheng, Y., and Kamholz, J. (1994). A novel cyclic AMP response element, CACTTGATC mediates forskolin induction of the myelin basic protein promoter in the rat Schwannoma line, D6P2T. J. Neurochem. *63*, 28-40.

Loring, J. F., and Erickson, C. A. (1987). Neural crest pathways in the trunk of the chick embryo. Dev. Biol. *121*, 220-236.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265-275.

Lue, R., Marfatia, S. M., Branton, D., and Chishti, A. H. (1994). Cloning and characterization of hdlg: the human homolog of the *Drosophila* discs-large tumor suppressor binds to protein 4.1. Proc. Natl. Acad. Sci. USA *91*, 9818-9822.

Lupski, J. R., Wise, C. A., Kuwano, A., Pentao, L., Parke, J. T., Glaze, D. G., Ledbetter, D. H., Greenberg, F., and Patel, P. (1992). Gene dosage is a mechanism for Charcot-Marie-Tooth disease type 1A. Nature Genet. *1*, 29-33.

Maggio, B., and Yu, R. K. (1992). Modulation by glycosphingolipids of membrane-membrane interaction induced by myelin basic protein and mellitin. Biochim. Biophys Acta. *112*, 105-114.

Manfioletti, G., Ruaro, M. E., Del Sal, G., Philipson, L., and Schneider, C. (1990). A growth arrest-specific (gas) gene codes for a membrane protein. Mol. Cell Biol. *10*, 2924-2930.

Marfatia, S. M., Cabral, J. H. M., Lin, L. H., Hough, C., Bryant, P. J., Stolz, L., and Chishti, A. H. (1996). Modular organization of the PDZ domains in the human discs-large protein suggests a mechanism for coupling PDZ domain-binding proteins to ATP and the membrane cytoskeleton. J. Cell BIol. *135*, 753-766.

Marrosu, M. G., Vaccargiu, S., Marrosu, G., Vanelli, A., Cianchetti, C., and Muntoni, F. (1997). A novel point mutation in the peripheral myelin protein-22 (PMP22) gene associated with Charcot-Marie-Tooth disease type 1A. Neurology *48*, 489-493.

Martenson, R. E. (1992). Myelin: Biology and Chemistry: CRC Press.

Martenson, R. E., Law, M., and Deibler, G. E. (1983). Identification of multiple in vivo phosphorylation sites in rabbit myelin basic protein. J. Biol. Chem. *258*, 930-937.

Martenson, R. E., and Uyemura, K. (1992). Myelin P2, a neuritogenic member of the family of cytoplasmic lipid-binding proteins. In Myelin: Biology and Chemistry, R. E. Martenson, ed.: CRC Press), pp. 509-528.

Martin, J. R., and Webster, H. de. F. (1973). Mitotic nerve cells in developing nerve: Their changes in shape, fine structure and axon relationships. Dev. Biol. *32*, 417-425.

Martini, R., Mohajeri, M. H., Kasper, S., Giese, K. P., and Schachner, M. (1995). Mice doubly deficient in the genes for P0 and myelin basic protein show that both proteins contribute to the formation of the major dense line in peripheral nerve myelin. J. Neurosci. *15*, 4488-4495.

Martini, R., and Schachner, M. (1988). Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, and myelin-associated glycoprotein) in regenerating adult mouse sciatic nerve. J. Cell Biol. *106*, 1735-1746.

Martyn, C. (1991). Epidemiology. In McAlpines Multiple Sclerosis, W. B. Matthews, ed.: Churchill Livingstone).

Matsunami, N., Smith, B., Ballard, L., Lensch, M. W., Robertson, M., Albertson, H., Hanemann, C. O., Muller, H. W., Bird, T. D., White, R., and Chance, P. F. (1992). Peripheral myelin protein-22 maps in the duplication in chromosome 17p11.2 associated with Charcot-Marie-tooth 1A. Nature Genet. *1*, 159-165.

Matthews, M. A. (1968). An electron microscopic study on the relationship between axon diameter and the initiation of myelin production in the peripheral nervous system. Anat. Rec. *161*, 337-346.

Mendz, G. L. (1992). Structure and molecular interactions of myelin basic protein and its antigenic peptides. In Myelin: Biology and Chemistry, R. E. Martenson, ed.: CRC Press), pp. 277-281.

Mikoshiba, K., Kohsaka, K., Takamatsu, K., and Tsukada, Y. (1981). Neurochemical and morphological studies on the myelin of the peripheral nervous system from shiverer mutant mice: absence of basic proteinscommon to central nervous system. Brain Res. *204*, 455-458.

Mikoshiba, K., Okano, H., Tamura, T., and Ikenaka, K. (1991). Structure and function of myelin protein genes. Annu. Rev. Neurosci. *14*, 201-217.

Miller, S. L., Benjamins, J. A., and Morell, P. (1977). Metabolism of glycerophospholipids of myelin and microsomes in rat brain: reutilization of precursors. J. Biol Chem.

Milner, R. J., Lai, C., Nave, K., Montag, D., and Farber, L. (1990). Organization of myelin protein genes: myelin associated glycoprotein. In Myelination and Dysmyelination, I. D. Duncan, R. P. Skoff and D. R. Colman, eds. (New York: Ann. NY. Acad. Sci.).

Mirsky, R., Dubois, C., Morgan, L., and Jessen, K. R. (1990). O4 and A007-sulfatide antibodies bind to embryonic Schwann cells prior to the appearance of galactocerebroside; regulation of the antigen by axon-Schwann cell signals and cAMP. Development *109*, 105-116.

Mirsky, R., and Jessen, K. R. (1996). Schwann-cell development, differentiation and myelination. Curr. Opin. Neurobiol. *6*, 89-96.

Montag, D., Giese, K. P., Bartsch, U., Martini, R., Lang, Y., Bluthmann, H., Karthigasan, J., Kirschner, D. A., Wintergerst, E. S., Nave, K. A., Zielasek, J., Toyka, K. V., Lipp, H. V., and Schachner, M. (1994). Mice deficient for the myelin-associated glycoprotein show subtle abnormalities in myelin. Neuron *13*, 229-246.

Monuki, E. S., Kuhn, R., and Lemke, G. (1993). Repression of the myelin P0 gene by the POU transcription factor SCIP. Mechanisms of Dev. *42*, 15-32.

Monuki, E. S., Weinmaster, G., Kuhn, R., and Lemke, G. (1989). SCIP: A glial POU domain gene regulated by cyclic AMP. Neuron *3*, 783-793.

Moore, G. P. (1983). Slipped-mispairing and the evolution of introns. TIBS *8*, 411-414.

Morell, P. (1984). Myelin (New York: Plenum Press).

Morell, P., Quarles, R. H., and Norton, W. T. (1989). Formation, structure, and biochemistry of myelin. In Basic Neurochemistry: Molecular, Cellular and Medical Aspects, G. J. Siegel, B. W. Agranoff, R. W. Albers and P. B. Molinoff, eds. (New York: Raven), pp. 109-136.

Morgan, L., Jessen, K. R., and Mirsky, R. (1991). The effects of CAMP on differentiation of cultured Schwann cells:progression from an early phenotype (O4+) to a myelin phenotype (P0+, GFAP-, NCAM-, NGF-receptor-) depends on growth inhibition. J. Cell Biol. *112*, 457-467.

Morgan, L., Jessen, K. R., and Mirsky, R. (1994). Negative regulation of the P0 gene in Schwann cells: suppression of P0 mRNA and protein induction in cultured Schwann cells by FGF2 and TGF beta 1, TGF beta 2 and TGF beta 3. Development *120*, 1399-1409.

Muller, B. M., Kistner, B. M., Veh, R. W., Cases-Langoff, C., Becker, B., Gudelfinger, E. D., and Garner, C. C. (1995). Molecular characterization and spatial distribution of SAP97, a novel presynaptic protein homologous to SAP90 and the *Drosophila* Discs-Large tumor suppressor protein. J. Neurosci. *15*, 2354-2366.

Muller, H. W. (1982). Dissociation of CNPase dimer into catalytically active monomers by 1,4-thiothreitol and urea. FEBS Lett. *144*, 77-80.

Muller, H. W., Clapshaw, P. A., and Seifert, W. (1981). Two molecular forms of the isolated brain enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase. FEBS Lett. *131*, 37-40.

Nakano, R., Fujita, N., Sato, S., Inuzuka, T., Sakimura, K., Ishiguro, H., Mishina, M., and Miyatake, T. (1991). Structure of mouse myelin-associated glycoprotein gene. Biochim. Biophys. Res. Commun. *178*, 282-290.

Naora, H., Miyahara, K., and Curnow, R. N. (1987). Origin of noncoding DNA sequences: molecular fossils of genome evolution. Proc. Natl. Acad. Sci. USA *84*, 6195-6199.

Napolitano, L. M., and Scallen, T. J. (1969). Observations on the fine structure of peripheral nerve myelin. Anat. Rec. *163*, 1.

Narayanan, V., Barbosa, E., Randall, R., and Tennekoon, G. I. (1991). Structure of the mouse myelin P2 protein gene. J. Biol. Chem. *263*, 8332-8337.

Nave, K. A. (1994). Neurological mouse mutants and the genes of myelin. J. Neurosci. Res. *38*, 607-612.

Navon, R., Seifried, B., Galon, N. S., and Sadeh, M. (1996). A new point mutation affecting the fourth transmembrane domain of PMP22 results in severe , de novo Charcot-Marie-Tooth disease. Hum. Genet. *97*, 685-687.

Nelles, E., Butzler, C., Jung, D., Temme, A., Gabriel, H. D., Dahl, U., Traub, O., Stumpel, F., Jungermann, K., and Zielasek, J. (1996). Defective propagation of signals generated by sympathetic nerve stimulation in the liver of connexin 32-deficient mice. Proc. Natl. Acad. Sci. USA *93*, 9565-9570.

Neuberger, T. J., and De Vries, G. H. (1992). Axonal contact as a determinant of Oligodendrocyte ans Schwann cell function. In Myelin: Biology and Chemistry, R. E. Martenson, ed.: CRC Press).

Nevins, J. R., and Chen-Kiang, S. (1981). Processing of adenovirus nuclear RNA to mRNA. Adv. Virus Res. *26*, 1-35.

Newman, S., Kitamura, K., and Campagnoni, A. T. (1987). Identification of a cDNA coding for a fifth form of myelin basic protein in mouse. Proc. Natl. Acad. Sci. USA *84*, 886-890.

Nicholson, G., Valentijn, L. J., Cherryson, A. K., Kennerson, M. L., Bragg, T. L., Dekroon, R. M., Ross, D. A., Pollard, J. D., McLeod, J. G., Bolhuis, P. A., and Baas, F. (1994). A frame shift mutation in the PMP22 gene in hereditary neuropathy with liability to pressure palsies. Nature genet. *6*, 263-266.

Norton, W. T., and Cammer, W. (1984). Isolation and Characterization of Myelin. In Myelin, P. Morell, ed. (New York: Plenum Press).

O'Brien, J. S., Sampson, E. I., and Stern, M. B. (1967). Lipid composition of myelin from the peripheral nervous system: intradural spinal roots. J. Neurochem. *14*, 357.

Ochoa, J. (1976). The unmyelinated nerve fibre. In The Peripheral Nerve, D. N. Landon, ed. (London: Chapman and Hall), pp. 19-76.

Olafson, R. W., Drummon, G. I., and Lee, J. F. (1969). Studies on 2'3'-cyclic nucleotide 3'-phosphohydrolase from brain. Can. J. Biochem. *47*, 961-966.

Ong, R. L., and Yu, R. K. (1884). Interaction of ganglioside GM1 and myelin basic protein studied by 12C and1H nuclear magnetic resonance. J. Neurosci. Res. *12*, 377-393.

Pannese, E., Ledda, M., and Matsuda, S. (1988). Nerve fibres with myelinated and unmyelinated portions in dorsal spinal roots. J. Neurocytol *17*, 693-700.

Pareek, S., Suter, U., Snipes, G. J., Welcher, A. A., Shooter, E. M., and Murphy, R. A. (1993). Detection and processing of peripheral myelin protein PMP22 in cultured Schwann cells. J. Biol. Chem. *268*, 10372-10379.

Patel, P. I., Roa, B. B., Welcher, A. A., Schoener-Scott, R., Trask, B. J., Pentao, L., Snipes, G. J., Garcia, C. A., Francke, U., Shooter, E. M., Lupski, J. R., and Suter, U. (1992). The gene for the peripheral myelin protein PMP22 is a candidate for Charcot-Marie-Tooth disease type 1a. Nature Genet. *1*, 159-165.

Peters, A., and Muir, A. R. (1959). The relationship between axons and Schwann cells during development of peripheral nerves in the rat. Q. J. Exp. Physiol *64*, 117-125.

Peters, A., and Vaughn, J. E. (1970). Morphology and development of the myelin sheath. In Myelination, A. N. Davison and A. Peters, eds. (Illinois: Charles C. Thomas).

Peterson, R. G., and Grevner, R. W. (1978). Morphological localization of PNS myelin proteins. Brain Res. *152*, 17-29.

Peterson, R. G., and Pease, D. C. (1972). Myelin embedded in polymerized glutaraldehyde-urea. J. Ultrastruc. Res. *41*, 115-120.

Pleasure, D., Krieder, B., Shuman, S., and Sobue, G. (1985). Tissue culture studies of Schwann cell proliferation and differentiation. Dev. Neurosci. *7*, 364-373.

Poduslo, J. F. (1984). Regulation of myelination: biosynthesis of the major myelin glycoprotein by Schwann cells in the presence and absence of myelin assembly. J. Neurochem. *42*, 493-503.

Politis, M. J., Sternberger, N., Ederle, K., and Spencer, P. S. (1982). Studies on the control of myelinogenesis. J. Neurosci. 2, 1252-1266.

Poltorak, M., Sadoul, R., Keilhauer, G., Landa, C., Fahrig, T., and Schachner, M. (1987). The myelin associated glycoprotein (MAG), a member of the L2/HNK-1 family of neural adhesion molecules is inolved in neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte interaction. J.Cell Biol. *105*, 1893-1899.

Poulat, F., de Santa Barbara, P., Desclozeaux, M., Soullier, S., Moniot, B., Bonneaud, N., Boizet, B., and Berta, P. (1997). The human testis determining factor *SRY* binds a nuclear factor containing PDZ preotein interaction domains. J. Biol. Chem. *272*, 7167-7172.

Pribyl, T. M., Campagnoni, C. W., Kampf, K., Handley, V. W., McMahon, J., and Campagnoni, A. T. (1993). The human myelin basic protein gene is included within a 179-kilobase transcription unit: expression in the immune and central nervous system. Proc. Natl. Acad. Sci. USA *90*, 10695-10699.

Pringle, N. P., and Richardson, W. D. (1993). A singularity of PDGF alpha-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage. Development *117*, 525-533.

Puckett, C., Hudson, L., Ono, K., Friedrich, V., Benecke, J., Dubois, D. M., and Lazzarini, R. A. (1987). Myelin-specific proteolipid protein is expressed in myelinating Schwann cells but is not incorporated into myelin sheaths. J. Neurosci. Res. *18*. 511-518.

Quarles, R. H., Colman, D. R., Salzer, J. L., and Trapp, B. D. (1992). Myelin associated glycoprotein: structure-function relationships and involvement in neurological diseases. In Myelin: Biology and Chemistry, R. E. Martenson, ed.: CRC Press), pp. 413-448.

Radin, N. S. (1983). Sulfatides. In Handbook of Neurochemistry, A. Lajtha, ed. (New York: Plenum Press).

Raine, C. S. (1984). Morphology of myelin and myelination. In Myelin, P. Morell, ed. (New York: Plenum Press), pp. 1-50.

Raine, C. S., and Schaumburg, H. H. (1984). Neuropathology of myelin diseases. In Myelin, P. Morell, ed. (New York: Plenum), pp. 271-323.

Ranvier, M. L. (1871). Contributions al'histologie et a la physiologie des nerfs peripheriques. C. R. Acad. Sci. *73*, 11-18.

Ranvier, M. L. (1878). Lecons sur l'histologie du systeme nerveux (Paris: Librairie F. Savy).

Readhead, C., Popko, B., Takahashi, N., Shine, H. D., Saavedra, R. A., Sidman, R. L., and Hood, L. (1987). Expression of a myelin basic protein gene in transgenic shiverer mice: correction of the dysmyelinating phenotype. Cell *48*, 703-712.

Rickmann, M., Fawcett, J. W., and Keynes, R. J. (1985). The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. J. Embryol. Exp. Morph. *90*, 437-455.

Ritchie, J. M. (1987). Ion channels in neural membranes. Discussions Neurosci. *4*, 1-53.

Ritchie, J. M. (1984). Physiological basis of conduction in myelinated nerve fibres. In Myelin, P. Morell, ed. (New York: Plenum Press), pp. 117-145.

Roa, B. R., Garcia, C. A., Suter, U., Kulpa, D. A., Wise, C. A., Mueller, J., Welcher, A. A., Snipes, G. J., Shooter, E. M., Patel, P. I., and Lupski, J. R. (1993). Charcot-Marie-Tooth disease type 1A: Association with a spontaneous point mutation in the PMP22 gene. N. Engl. J. Med. *329*, 96-101.

Roach, A., Boylan, K., Horvath, S., Prusiner, S. B., and Hood, L. E. (1983). Charcterisation of cloned cDNA representing rat myelin basic protein: absence of expression in the brain of shiverer mutant mice. Cell *34*, 799-806.

Roach, A., Takahashi, N., Pravtcheva, D., Ruddle, F., and Hood, L. (1985). Chromosomal mapping of mouse myelin basic protein gene and structure and transcription of the partially deleted gene in shiverer mutant mice. Cell *42*, 149-155.

Rowe-Rendleman, C. L., and Eichberg, J. (1994). P0 phosphorylation in nerves from normal and diabetic rats: role of protein kinase C and turnover of phosphate groups. Neurochem. Res. *19*, 1023-1031.

Ruvkun, G., and Finney, M. (1991). Regulation of transcription and cell identity by POU domain proteins. Cell *64*, 475-478.

Saavedra, R. A., Fors, L., Aebersold, R. H., Arden, B., Horvath, S., Sanders, J., and Hood, L. (1989). The myelin proteins of the shark brain are similar to the myelin proteins of the mammalian peripheral nervous system. J. Mol. Evol. *29*, 149-156.

Sakamoto, Y., Kitamura, K., Yoshimura, K., Nishijima, T., and Uyemura, K. (1987). Complete amino acid sequence of P0 protein in bovine peripheral nerve myelin. J. Biol. Chem. *262*, 4208-4214.

Salzer, J. L., and Bunge, R. P. (1980). Studies on Schwann cell proliferation 1. An analysis in tissue culture of proliferation during development, Wallerian degeneration and direct injury. J. Cell Biol. *84*, 739-752.

Salzer, J. L., and Colman, D. R. (1989). Mechanisms of cell adhesion in the nervous system: role of the immunoglobulin gene superfamily. Dev. Neurosci. *11*, 377-390.

Salzer, J. L., Holmes, W. P., and Colman, D. R. (1987). The amino acid sequences of the myelin associated glycoproteins: homology to the immunoglobulin gene superfamily. J. Cell Biol. *104*, 957-965.

Salzer, J. L., Pedraza, L., Brown, M., Struyk, A., Afar, D., and Bell, J. (1990). Structure and function of the myelin associated glycoprotein. Ann. N.Y. Acad. Sci *605*, 302-312.

Sawadogo, M., and Van Dyke, M. W. (1991). A rapid method for the purification of deprotected oligodeoxynucleotides. Nucl. Acids Res. *19*, 674.

Schenone, A., Nobbio, L., Mandich, P., Bellone, E., Abbruzzese, M., Aymar, F., Mancardi, G. L., and Windebank, A. J. (1997). Underexpression of messenger RNA for peripheral myelin protein 22 in hereditary neuropathy with liability to pressure palsies. Neurology *48*, 455-449.

Scherer, S. S. (1997). The biology and pathobiology of schwann cells. Curr. Opin. Neurol. *10*, 386-397.

Scherer, S. S., Deschenes, S. M., Xu, Y.-T., Grinspan, J. B., Fischbeck, K. H., and Paul, D. L. (1995). Connexin 32 is a myelin-related protein in the PNS and CNS. J.Neurosci. *15*, 8281-8294.

Scherer, S. S., and Salzer, J. L. (1995). Axon-Schwann cell interactions in peripheral nerve regeneration. In Glial Cell Development, K. R. Jessen and W. D. Richardson, eds.: Bios Scientific Publishing).

Scherer, S. S., Xu, Y. T., Bannerman, P. G. C., Sherman, D. L., and Brophy, P. J. (1995). Periaxin expression in myelinating Schwann cells: Modulation by axon-glial interactions and polarized localization during development. Development *121*, 4265-4273.

Schober, R., Itoyama, J., Sternberger, N. H., Trapp, B. D., Richardson, E. P., Ashbury, A. K., Quarles, R. H., and Webster, H. d. (1981). Immunocytochemical study of P0 glycoprotein, P1, and P2 basic proteins and myelin associated glycoprotein (MAG) in lesions of idiopathic polyneuritis. Neuropath. and Applied Neurobiol. *7*, 437-451.

Schöler, H. R. (1991). Octamania: The POU factors in murine development. Trends in Genetics *7*, 323-329.

Scolding, N. J., Zajicek, J. P., Wood, N., and Compston, D. A. S. (1994). The pathogenesis of demyelinating disease. Prog. Neurobiol. *43*, 143-173.

Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D. (1989). The metabolic basis of inherited disease (New York: McGraw Hill).

Senepathy, P. (1986). Origin of eukaryotic introns: a hypothesis based on codon distribution statistics in genes, and its implications. Proc. Natl. Acad. Sci. USA *83*, 2133-2137.

Shapiro, M. B., and Senepathy, P. (1987). RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucl. Acids Res. *15*, 7155-7175.

Shieh, B.-H., and Zhu, M.-Y. (1996). Regulation of the TRP Ca^{2+} channel by INAD in Drosophila photoreceptors. Neuron *16*, 991-998.

Shuman, S., Hardy, M., and Pleasure, D. (1986). Immunochemical characterization of peripheral nervous system myelin 170,000-Mr glycoprotein. J. Neurochem. *47*, 811-818.

Shuman, S., Hardy, M., Sobue, G., and Pleasure, D. (1988). A cyclic AMP analogue induces the synthesis of a myelin-specific glycoprotein by cultured Schwann cells. J. Neurochem. *50*, 190-194.

Simpson, S. A., and Young, J. Z. (1945). Regeneration of fibre diameter after cross-union of visceral and somatic nerves. J. Anat. *79*, 48-65.

Skre, H. (1974). Genetic and clinical aspects of Charcot-Marie-Tooth's disease. Clin. Genet *6*, 98-118.

Smith, M. (1983). Peripheral nervous system myelin: properties and metabolism. In Handbook of Neurochemistry, A. Lajtha, ed. (New York: Plenum Press), pp. 201-220.

Smith, M. E. (1968). The turnover of myelin in the adult rat. Biochim. Biophys. Acta. *164*, 285.

Smith, M. E., and Curtis, B. M. (1979). Frog sciatic nerve myelin:a chemical characterisation. J. Neurochem. *33*, 447.

Smith, R. (1992). The basic proteins of CNS myelin:its structure and ligand binding. J. Neurochem. *59*, 1589-1608.

Snipes, G. J., Suter, U., Welcher, A. A., and Shooter, E. M. (1992). Characterization of a novel peripheral nervous system myelin protein (PMP-22/SR13). J. Cell Biol. *117*, 225-238.

Sobue, G., and Pleasure, D. (1984). Schwann cell galactocerebroside induced by derivatives of adenosine 3',5'-monophosphate. Science *224*, 72-74.

Sommer, I., and Schachner, M. (1981). Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. Dev. Biol. *83*, 311-327.

Spagnol, G., Williams, M., Srinvasan, J., Golier, J., Bauer, D., Lebo, R. V., and Latov, N. (1989). Molecular cloning of human myelin associated glycoprotein. J. Neurosci. Res *24*, 137-142.

Spreyer, P., Kuhn, G., Hanemann, C. O., Gillen, C., Schaal, H., Kuhn, R., Lemke, G., and Muller, H. W. (1991). Axon-related expression of a Schwann cell transcript that is homologous to a 'growth arrest-specific' gene. EMBO J. *10*, 3661-3668.

Sprinkle, T. J. (1989). 2',3'-cyclic nucleotide 3'-phosphodiesterase, an oligodendrocyte-Schwann cell and myelin associated enzyme of the nervous system. Crit. Rev. Neurobiol. *4*, 235-301.

Sprinkle, T. J., McMorris, A., Yoshino, J., and De, V. G. (1985). Differential expression of 2':3'-cyclic nucleotide 3'-phosphodiesterase in cultured central, peripheral, and extraneural cells. Neurochem. Res. *10*, 919-931.

Spritz, N., Singh, H., and Geyer, B. (1973). Myelin from human peripheral nerves: quantitative and qualitative studies in two age groups. J. Clin. Invest. *52*, 520.

Stampfli, R. (1981). Overview of studies on the physiology of conduction in myelinated nerve fibres. Adv. Neurol. *31*, 11.

Stemple, D. L., and Anderson, D. J. (1992). Isolation of a stem cell for neurons and glia from the mammalian neural crest. Cell *71*, 973-985.

Sternberger, N. H., Quarles, R. H., Itoyama, Y., and Webster, H. d. (1979). Myelina associated glycoprotein demonstrated immunocytochemically in myelin and non-myelin forming cells of developing rats. Proc. Natl. Acad. Sci. USA *76*, 1510-1514.

Streicher, R., and Stoffel, W. (1989). The organization of the human myelin basic protein gene - comparison with the mouse gene. Biol. Chem. Hoppe-Seyler *370*, 503-510.

Sutcliffe, J. G. (1987). The genes for myelin. Trends in Genetics *3*, 73-76.

Suter, U., and Snipes, G. J. (1995). Biology and genetics of hereditary motor and sensory neuropathies. Annu. Rev. Neurosci. *18*, 45-75.

Suter, U., Snipes, G. J., Schoener-Scott, R., Welcher, A. A., Pareek, S., Lupski, J. R., Murphy, R. A., Shooter, E. M., and Patel, P. I. (1994). Regulation of tissue-specific expression of alternative peripheral myelin protein-22 (PMP22) gene transcripts by two promoters. J. Biol. Chem. *269*, 25795-25808.

Suter, U., Welcher, A. A., Ozcelik, T., Snipes, G. J., Kosaras, B., Francke, U., Billings-Gagliardi, S., Sidman, R. L., and Shooter, E. M. (1992). Trembler mouse carries a point mutationin a myelin gene. Nature *356*, 241-243.

Suzuki, M., Kitamura, K., Sakamoto, Y., and Uyemura, K. (1982). The complete amino acid sequence of the human P2 protein. J. Neurochem. *39*, 1759-1762.

Suzuki, M., Sakamoto, Y., Kitamura, K., Fukunaga, K., Yamamoto, H., Miyamoto, E., and Uyemura, K. (1990). Phosphorylation of P0 glycoprotein in peripheral nerve myelin. J. Neurochem. *55*, 1966-1971.

Takahashi, N., Roach, A., Teplow, D. B., Prusiner, S. B., and Hood, L. (1985). Cloning and charcaterisation of the myelin basic protein gene from mouse: one gene can encode both 14 kd and 18.5 kd MBPs by alternate use of exons. Cell *42*, 139-148.

Takeuchi, M., Hata, Y., Hirao, K., Toyoda, A., Irie, M., and Takai, Y. (1997). SAPAPS: A family of PSD-95/SAP90-associated proteins localized at postsynaptic density. J. Biol. Chem. *18*, 11943-11951.

Taniuchi, M., Clark, H. B., and Johnson, E. M. (1986). Induction of nerve growth factor receptors in Schwann cells after axotomy. Proc. Natl. Acad. Sci. USA *83*, 4094-4098.

Tetzloff, S. U., and Bizzozero, O. A. (1993). Proteolipid protein from the peripheral nervous system also contains covalently bound fatty acids. Biochim. Biophys. Res. Commun. *193*, 1304-1310.

Timmerman, V., Nelis, E., Van Hul, W., Nieuwenhuijsen, B. W., Chen, K. L., Ben Othman, K., Cullen, B., Leachz, R. J., Hanemann, C. O., De Jonghe, P., Raeymaekers, P., van Ommen, G. J., Martin, J. J., Muller, H. W., Vance, J. M., Fischbeck, K. H., and Van Broeckhoven, C. (1992). The peripheral myelin protein gene PMP22 is contained within the Charcot-Marie-Tooth disease type 1a duplication. Nature Genet. *1*, 171-175.

Toews, A., and Morell, P. (1987). Posttranslational modification of myelin proteins. In A Multidisciplinary Approach to Myelin Diseases, C. S. Crescenzi, ed. (New York: Plenum Press).

Topilko, P., Murphy, P., and Charnay, P. (1996). Embryonic development of Schwann cells: multiple roles for neuregulins along the pathway. Mol. Cell. Neurosci. *8*, 71-75.

Towbin, H., Staehlin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose: procedure and some applications. Proc. Natl. Acad. Sci. USA *76*, 4350-4354.

Trapp, B. D., Dubois-Dalcq, M., and Quarles, R. H. (1984). Ultrastructural localization of P2 protein in actively myelinating Schwann cells. J. Neurochem. *43*, 944-948.

Trapp, B. D., Hauer, P., and Lemke, G. (1988). Axonal regulation of myelin protein mRNA levels in actively myelinating Schwann cells. J. Neurosci. *8*, 3515-3521.

Trapp, B. D., and Quarles, R. H. (1982). Presence of the myelin-associated glycoprotein correlates with alterations in the periodicity of peripheral myelin. J. Cell Biol. *92*, 877-882.

Trapp, B. D., Quarles, R. H., and Griffin, J. W. (1984). Myelin associated glycoprotein and myelinating Schwann cell-axon interaction in chronic B, B'-iminodipropionitrile neuropathy. J. Cell Biol. *98*, 1272-1278.

Tropak, M. B., Johnson, P. W., Dunn, R. J., and Roder, J. C. (1988). Differential splicing of MAG transcripts during CNS and PNS development. Mol. Brain res. *4*, 143-155.

Tsukada, Y., and Kurihara, T. (1992). 2',3'-cyclic nucleotide 3'-phosphodiesterase: molecular characterization and possible functional significance. In Myelin: Biology and Chemistry, R. E. Martenson, ed.: CRC Press), pp. 449-480.

Turner, R. S., Jen Chou, C. H., Mazzei, G. J., Dembure, P., and Kou, J. F. (1984). Phospholipid-sensitive Ca2+ -dependent protein kinase preferentially phosphorylates serine-115 of bovine myelin basic protein. J. Neurochem. *43*, 1257-1264.

Uyemura, K., Suzuki, M., Sakamoto, Y., and Tanaka, S. (1987). Structure of P0 protein: homology to immunoglobulin superfamily. Biomed. Res. *8*, 353-357.

Uyemura, K., Yoshimura, K., Suzuki, M., and Kitamura, K. (1984). Lipid binding activities of the P2 protein in peripheral nerve myelin. Neurochem. Res. *9*, 1509-1514.

Valentijn, L. J., Baas, F., and Wolterman, R. A. (1992). Identical point mutations of PMP22 in trembler-J mouse and Charcot-Marie-Tooth disease type 1a. Nature Genet. *2*, 288-291.

Valentijn, L. J., Bolhuis, P. A., Zorn, I., Hoogendijk, J. E., van den Bosch, N., Hensels, G. W., Stanton, V. P., Housman, D. E., Fischbeck, K. H., Ross, D. A., Nicholson, G. A., Meershoek, E. J., Dauwerse, H. G., van Ommen, G. J., and Baas, F. (1992). The peripheral myelin gene PMP22/gas3 is duplicated in Charcot-Marie-Tooth disease type 1a. Nat. Genet. *1*, 166-170.

Vallat, J. M., Sindou, P., Preux, P. M., Tabaraud, F., Milor, A. M., Couratier, P., Leguren, E., and Brice, A. (1996). Ultrastructural PMP22 expression in inherited peripheral neuropathies. Ann. Neurol. *39*, 813-817.

Vance, J. M., Barker, D., Yamaoka, L. H., Stajich, J. M., Loprest, L., Hung, W. Y., Fischbeck, K., Roses, A. D., and Pericak-Vance, M. A. (1991). Localization of Charcot-Marie-Tooth disease type 1a (CMT1A) to chromosome 17p11.2. Genomics *9*, 623-628.

Vance, J. M., Nicholson, G. A., and Yamaoka, L. H. (1989). Linkage of Charcot-Marie-Tooth neuropathy type 1a to chromosome 17. Exp. Neurol. *104*, 186-189.

Veerkamp, J. H., Peeters, R. A., and Maatman, H. J. (1991). Structural and functional features of different types of cytoplasmic fatty acid-binding proteins. Biochim. Biophys Acta *108*, 1-24.

Vogel, U. S., and Thompson, R. J. (1988). Molecular structure, localization, and possible functions of the myelin-associated enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase. J. Neurochem. *50*, 1667-1677.

Warbrick, E. (1997). Two's company, three's a crowd: the yeast two hybrid system for mapping molecular interactions. Structure *5*, 13-17.

Warner, L. E., Hilz, M. J., Appel, S. H., Killian, J. M., Watters, G. V., Wheeler, C., Witt, D., Bodell, A., Nelis, E., Van Broeckhoven, C., and Lupski, J. R. (1996). Clinical phenotypes of different MPZ (P0) mutations may include Charcot-Marie-Tooth type 1B, Dejerine-Sottas, and congenital hypomyelination. Neuron *17*, 451-460.

Waxman, S. G., and Ritchie, J. M. (1985). Organisation of ion channels in the myelinated nerve fibre. Science *228*, 1502-1507.

Webster, H. de F. (1971). The geometry of peripheral myelin sheaths during their formation and growth in rat sciatic nerve. J. Cell Biol. *48*, 348-367.

Webster, H. de F., and Favilla, J. T. (1984). Development of peripheral nerve fibres. In Peripheral Neuropathy, P. J. Dyck, P. K. Thomas, E. H. Lambert and R. Bunge, eds. (Philadelphia: W. B. Saunders), pp. 329-359.

Webster, H. de F., Martin, J. R., and O'Connell, M. F. (1973). The relationship between interphase Schwann cells and axons before myelination: a quantitative electron microscopic study. Dev. Biol. *32*, 401-412.

Wegner, M., Drolet, D. W., and Rosenfeld, M. G. (1993). POU-domain proteins: structure and function of developmental regulators. Curr. Opin. Cell Biol. *5*, 488-498.

Weinberg, H. J., and Spencer, P. S. (1976). Studies on the control of myelinogenesis II. Evidence for neuronal regulation of myelin production. Brain Res. *113*, 363-378.

Welcher, A. A., Suter, U., De Leon, M., Snipes, G. J., and Shooter, E. M. (1991). A myelin protein is encoded by the homologue of a growyh arrest-specific gene. Proc. Natl. Acad. Sci. USA *88*, 7195-7199.

Weston, J. A. (1963). A radiographic analysis of the migration and localization of trunk neural crest cells in the chick. Dev. Biol. *6*, 279-310.

Wiegandt, H. (1982). In Advances in Neurochemistry, B. W. Agranoff and M. H. Aprison, eds. (New York: Plenum Press), pp. 149-223.

Willison, H. J., Ilyas, A. I., O'Shannessy, D. J., Pulley, M. J., Trapp, B. D., and Quarles, R. H. (1987). Myelin-associated glycoprotein and related glycoconjugates in developing cat peripheral nerve: a correlative biochemical and morphometric study. J. Neurochem. *49*, 1853-1862.

Willott, E., Balda, M. S., Fanning, A. S., Jameson, B., Van Itallie, C., and Anderson, J. (1993). The tight junction protein ZO-1 is homologous to the Drosophila discs-large tumor suppressor protein of septate junctions. Proc. Natl. Acad. Sci. USA *90*, 7834-7838.

Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., Cooper, J., Coulson, A., Craxton, M., Dear, S., Du, Z., and Durbin, R. (1994). 2.2 Mb of contiguous sequence from chromosome III of C. elegans. Nature *368*, 32-38.

Wong, M. H., and Filbin, M. T. (1996). Dominant-negative effect on adhesion by myelin P0 protein truncated in its cytoplasmic domain. J. Cell Biol. *134*, 1531-1541.

Wood, J. G., and Dawson, R. M. C. (1973). A major myelin protein of sciatic nerve. J. Neurochem. *21*, 717-721.

Wood, J. G., and Engel, E. L. (1976). Peripheral nerve glycoproteins and myelin fine structure during development of the rat sciatic nerve. J. Neurocytol. *5*, 605-615.

Wood, J. G., and McLaughlin, J. (1975). The visualization of concavalin A binding sites in the intraperiod line of rat sciatic nerve myelin. J. Neurochem *24*, 233-235.

Wood, P. M., and Bunge, R. P. (1975). Evidence that sensory axons are mitogenic for Schwann cells. Nature *256*, 662-664.

Woods, D. F., and Bryant, P. J. (1991). The Discs-Large tumor suppressor gene of Drosophila encodes a guanylate kinase homolog localized at septate junctions. Cell *66*, 451-464.

Woods, D. F., and Bryant, P. J. (1993). ZO-1, DlgA and PSD-95/SAP90: homologous proteins in tight, septate, and synaptic cell junctions. Mech. Dev. *44*, 85-89.

Yisraeli, J., Adelstein, R. S., Melloul, D., Nudel, U., Yaffe, D., and Cedar, H. (1986). Muscle-specific activation of a methylated chimeric actin gene. Cell *46*, 409-416.

Yisraeli, J., and Szyf, M. (1984). Gene methylation patterns and expression, A. Razin, H. Cedar and A. D. Riggs, eds. (New York: Springer-Verlag).

You, K. H., Hseih, C. L., Hayes, C., Stahl, N., Francke, U., and Popko, B. (1991). DNA sequence, genomic organisation, and chromosomal localization of the mouse peripheral myelin protein zero gene:identification of polymorphic alleles. Genomics *9*, 751-757.

Zachowski, A., and Devaux, P. F. (1989). Bilayer asymmetry and lipid transport across biomembranes. Comments Mol. Cell Biophys. *6*, 63-70.

Zeller, N. K., Hunkeler, M. J., Campagnoni, A. T., Sprague, J., and Lazzarini, R. A. (1984). Characterization of mouse myelin basic protein messenger RNAs with a myelin basic protein cDNA clone. Proc. Natl. Acad. Sci. USA *81*, 18-22.

Zorick, T. S., and Lemke, G. (1996). Schwann cell differentiation. Curr. Opin. Cell Biol. *8*, 870-876.